

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/19443

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/02; C12N 15/82; A01H 5/00
US CL :536/23.1; 800/281, 298; 435/69.1, 419, 468

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 800/281, 298; 435/69.1, 419, 468

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST/DERWENT, GENBANK, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,777,201 A (POUTRE et al) 07 July 1998, especially col. 14-18.	1-5,9-11,13-15
Y		-----
Y	US 5,057,419 A (MARTIN et al) 15 October 1991, col. 7-11.	6-8,12,16-21
Y	STUKEY et al. The OLE1 Gene of <i>Saccharomyces cerevisiae</i> Encodes the delta 9 Fatty Acid Desaturase and Can Be Functionally Replaced by the Rat Stearoyl-CoA Desaturase. Gene. The Journal of Biological Chemistry. 25 November 1990, Vol. 265, No. 33, pages 20144-20149, especially page 20146.	1-21
		1-21

Further documents are listed in the continuation of Box C. See patent family annex.

•	Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A	document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
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P	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

12 NOVEMBER 1999

Date of mailing of the international search report

06 DEC 1999

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/19443

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	POLASHOCK et al. Expression of the Yeast delta-9 Fatty Acid Desaturase in Nicotiana tabacum. Plant Physiology. 1992, Vol. 100, pages 894-901, especially page 895.	1-5,9-11,13-15 -----
Y		6-8,12,16-21

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference RUT 97-0081/	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/19443	International filing date (day/month/year) 24 AUGUST 1999	Priority date (day/month/year) 24 AUGUST 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 4 sheets.

This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.
3. This report contains indications relating to the following items:
 - I Basis of the report
 - II Priority
 - III Non-establishment of report with regard to novelty, inventive step or industrial applicability
 - IV Lack of unity of invention
 - V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
 - VI Certain documents cited
 - VII Certain defects in the international application
 - VIII Certain observations on the international application

Date of submission of the demand 15 MARCH 2000	Date of completion of this report 28 DECEMBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  ELIZABETH F. McELWAIN
Facsimile No. (703) 305-3230	Telephone No. 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/19443

I. Basis of the report

1. With regard to the elements of the international application:*

 the international application as originally filed the description:pages 1-62, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____ the claims:pages 63-66, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____ the drawings:pages 1-2, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____ the sequence listing part of the description:pages 1-5, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is:

 the language of a translation furnished for the purposes of international search (under Rule 23.1(b)). the language of publication of the international application (under Rule 48.3(b)). the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

 contained in the international application in printed form. filed together with the international application in computer readable form. furnished subsequently to this Authority in written form. furnished subsequently to this Authority in computer readable form. The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished. The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.4. The amendments have resulted in the cancellation of: the description, pages NONE the claims, Nos. NONE the drawings, sheets/fig NONE5. This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. statement**

Novelty (N)

Claims 1-21 YES
Claims NONE NO

Inventive Step (IS)

Claims NONE YES
Claims 1-21 NO

Industrial Applicability (IA)

Claims 1-21 YES
Claims NONE NO**2. citations and explanations (Rule 70.7)**

Claims 1-21 lack an inventive step under PCT Article 33(3) as being obvious over Poutre et al or Polashock et al in view of Applicants' admitted state of the prior art.

Poutre et al and Polashock et al each teach transforming a plant with a yeast desaturase gene.

Neither Poutre et al or Polashock et al teach customizing the sequence for improved expression in a plant cytoplasm.

Applicants' admitted state of the prior art teaches that modifying sequences for improved expression in plants was known (see page 18, at least)

Given the recognition of those of ordinary skill in the art of the value of expressing a yeast desaturase in plant for the purpose of modifying fatty acid expression in the plant, it would have been obvious to one of ordinary skill in the art to use the methods of Poutre et al or Polashock et al and to modify these methods by substituting a gene that has been customized for expression in a plant cytoplasm.

----- NEW CITATIONS -----

NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C07H 21/02; C12N 15/82; A01H 5/00 and US Cl.: 536/23.1; 800/281, 298; 435/69.1, 419, 468

PCT

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091763,331 A/1638 (Kathy)

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 21/02, C12N 15/82, A01H 5/00		A1	(11) International Publication Number: WO 00/11012 (43) International Publication Date: 2 March 2000 (02.03.00)
<p>(21) International Application Number: PCT/US99/19443</p> <p>(22) International Filing Date: 24 August 1999 (24.08.99)</p> <p>(30) Priority Data: 60/097,586 24 August 1998 (24.08.98) US</p> <p>(71) Applicant (<i>for all designated States except US</i>): RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY [US/US]; Old Queens, Somerset Street, New Brunswick, NJ 08903 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): MARTIN, Charles, E. [US/US]; 3 Seely's Run, Somerset, NJ 08873 (US). MITCHELL, Andrew [GB/US]; 106 North 3rd Street, Highland Park, NJ 08904 (US).</p> <p>(74) Agents: REED, Janet, E. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103-2307 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: SYNTHETIC FATTY ACID DESATURASE GENE FOR EXPRESSION IN PLANTS</p> <p>(57) Abstract</p> <p>A synthetic fatty acid desaturase gene for expression in a multicellular plant is provided. The gene comprises a desaturase domain and a cyt b₅ domain, and is customized for expression in a plant cytoplasm. Methods for designing and making a synthetic fatty acid desaturase gene customized for expression in a plant cytoplasm are also provided.</p>			

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**SYNTHETIC FATTY ACID DESATURASE GENE
FOR EXPRESSION IN PLANTS**

This application claims priority to U.S. Provisional Application No. 60/097,586, filed August 24, 1998, the entirety of which is incorporated by reference herein.

5

FIELD OF THE INVENTION

This invention relates to the field of genetic engineering, and more particularly to transformation of plants with heterologous fatty acid desaturase genes 10 modified for optimum expression in plants.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application in order to more fully describe the state of 15 the art to which this invention pertains. The disclosure of each of these publications is incorporated by reference herein.

Alteration of fatty acid desaturation in plants is of interest to plant biologists and food scientists 20 alike, due to the influence of unsaturated fatty acids on the health benefits and flavors of foods, as well as the role of these molecules in plant biological processes. For a nation interested in healthy diet, the quality of fats and oils depends on their fatty acid composition, 25 with oils high in monounsaturated fatty acids (e.g., canola, olive) gaining popularity as new health benefits are discovered. Considering the flavors of plant foods, many flavor-producing compounds are derived from peroxidation of unsaturated fatty acids. Thus, efforts 30 are being made to produce plants with increased amounts

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of unsaturated fatty acids, preferably monounsaturated fatty acids.

In animal and fungal cells, monounsaturated fatty acids are aerobically synthesized from saturated fatty acids by a microsomal Δ -9 fatty acid desaturase that is membrane bound and cytochrome b_5 -dependent. A double bond is inserted between the 9- and 10-carbons of palmitoyl (16:0) and stearoyl (18:0) CoA to form palmitoleic (16:1) and oleic (18:1) acids. In the reaction mechanism, electrons are transferred from NADH-dependent cytochrome b_5 reductase, via the heme-containing cytochrome b_5 (Cyt b_5) molecule, to the Δ -9 fatty acid desaturase. The major form of cytochrome b_5 in animal, fungal and plant cells exists as an independent protein molecule that is anchored to the membrane by a short, carboxyl terminal, hydrophobic stretch of amino acids. The carboxyl terminal anchor orients the heme group of the Cyt b_5 on the membrane surface and allows it to translationally diffuse across the surface of the membrane. This property of lateral mobility allows this form of cytochrome b_5 to participate as an electron donor to a number of different proteins that catalyze a variety of metabolic reactions on the membrane surface, including fatty acid desaturases, various sterol biosynthetic enzymes and a variety of cytochrome P450 mediated reactions. While this contributes to the versatility of Cyt b_5 as an electron donor, it also implies that the major form of cytochrome b_5 shuttles between its redox partners by translational diffusion across the surface of the membrane (Strittmatter and Rogers, Proc. Natl. Acad. Sci. USA, 72: 2658-2661, (1975; Lederer, Biochimie 76: 674-692, 1994). Furthermore, this mechanism suggests that an independent, membrane bound cytochrome b_5 molecule

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can potentially limit the rate of the metabolic reaction, depending on its abundance, its location on the membrane surface, its proximity to the electron acceptor, and the rate at which it can move and orient itself to the acceptor on the membrane surface.

In plants, unsaturated fatty acids are formed and incorporated into complex lipids in two distinct cellular compartments. *De novo* fatty acid synthesis occurs almost exclusively in the plastids, producing the 10 saturated species 16:0-ACP (acyl carrier protein) and 18:0-ACP. 18:1-ACP is formed from 18:0-ACP in the plastid by a soluble, ferredoxin-dependent Δ -9 desaturase. These fatty acids are then shunted into one of two routes - a plastid-localized "prokaryotic" pathway 15 or a cytosolic/ER (endoplasmic reticulum) "eucaryotic" pathway - for further modification and acylation into glycerolipids (Somerville and Browse, *Science* **252**: 80-87, 1991). The acyl ACPS that are shunted into the prokaryotic pathway remain within the plastid and are 20 used for the synthesis of phosphatidic acid and further conversion to chloroplast glycerolipids. The fatty acyl groups of those lipids may be further desaturated by plastid desaturases that also use ferrodoxin as the electron donor.

25 Acyl-ACPs that are shunted into the eukaryotic pathway are converted to free fatty acids, transported across the chloroplast membrane into the cytoplasm where they are converted to acyl CoA thioesters by acyl CoA synthetase. Those fatty acids are then converted to 30 cytoplasmic/ER phosphatidic acid which can then be converted to membrane glycerophospholipids, or storage lipids, in the form of triacylglycerols and sterol esters that are the major components of plant oils.

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Most polyunsaturated 18-carbon plant fatty acids appear to be formed in the cytosol by the ER-bound desaturases (Table 1). Once the 18:1 fatty acid is incorporated into phospholipid, an ER-bound desaturase 5 can catalyze the formation of a Δ -12 double bond in the fatty acyl chain to form Δ -9,12 18:2. Other ER bound desaturase enzymes can act on 18:2 to introduce a Δ -15 double bond to form Δ 9,12,15 18:3. These desaturase are thought to be similar to animal and fungal desaturases 10 because they are membrane bound and appear to require a cytochrome b_5 -mediated electron transport chain.

TABLE 1:

Plant	Gene	Desaturase Type	Primary Activity	b5 chimera	Reference
Arabidopsis	FAD2	Δ 12, microsomal	18:1->18:2	no	Okuley J. et al. Plant Cell <u>6</u> : 147-158, 1994
Arabidopsis	FAD3	Δ 15, microsomal	18:2->18:3	no	Shah S. & Z. Xin, Plant Physiol. <u>114</u> : 1533-1539, 1997
Nicotiana tabacum	NtFA D3	Δ 15, microsomal	18:2->18:3	no	Hamada T. et al. Plant & Cell. Physiol. <u>37</u> : 606-611, 1996, Hamada T. et al. Transgenic Res. <u>5</u> : 115-121, 1996
Soybean	FAD 2-1	Δ 12, microsomal, developing seeds	18:1->18:2	no	Heppard E.P. et al. Plant Physiol. <u>110</u> : 311-319, 1996
Soybean	FAD 2-2	Δ 12, microsomal developing seeds and vegetative tissues	18:1->18:2	no	Heppard, E.P. et al. 1996, <i>supra</i>

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Borage		Δ-6	18:2 (9,12)-18:3 (6,9,12)	yes, N-terminal	Sayanova et al. Proc. Natl. Acad. Sci. USA 94: 4211- 4216, 1997
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The conversion of saturated fatty acyl chains to monounsaturated species in plants appears to be confined to the chloroplasts. No Δ-9 desaturase activity has been identified in the cytoplasm or endoplasmic reticulum of plants. The soluble plant chloroplast Δ-9 desaturase is highly specific for 18:0-ACP as a substrate and does not desaturate 16:0-ACP (Somerville and Browse, *supra*). As a result, only a small amount of 16:1 is present in most higher plants, while the pool of 16:0 is concomitantly larger due to its disfavor as a substrate for the plant desaturase. By comparison, a larger amount of 18:1 is found in higher plant cells, with a correspondingly lesser amount of 18:0. Thus, for the purpose of increasing the concentration of mono-unsaturated lipids in a plant, the 16:0 fatty acid constitutes a significant pool of available substrate that is under-utilized by the endogenous plant desaturase.

In contrast to the plant Δ-9 desaturase, fungal and animal Δ-9 desaturases efficiently convert a wide range of saturated fatty acids with differing hydrocarbon chain lengths to monounsaturated fatty acids. The *Saccharomyces cerevisiae* enyzme, for example, efficiently desaturates even and odd chain fatty acyl CoA substrates from 13 carbons to 19 carbons in length. A broad functional homology exists among various Cyt b₅-dependent desaturases, as evidenced, for example, by the successful expression of the rat Δ-9 desaturase in yeast (Stukey et al., *J. Biol. Chem.* 265: 20144-20149, 1990).

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The rat and yeast Δ -9 desaturase genes have been expressed in plants: both the rat and the yeast genes have been expressed in tobacco (Grayburn et al., BioTechnology 10: 675-678, 1992 (rat); Polashock et al., 5 Plant Physiol. 100: 894-901, 1992 (yeast), and the yeast gene has also been expressed in tomato (Wang et al., J. Agric. Food Chem. 44: 3399-3402, 1996). The yeast Δ -9 desaturase has been shown to function in tobacco and tomato, leading to increases in the level of 10 monounsaturated fatty acids (both 16:1 and 18:1) and other compounds derived from monounsaturated fatty acids (e.g., polyunsaturated fatty acids, hexanal, 1-hexanol, heptanal, *trans*-2-octenal) (Polashock et al., *supra*; Wang et al; *supra*). Expression of the rat desaturase also led 15 to an increase in monounsaturated 16- and 18-carbon fatty acids (Grayburn et al., *supra*).

From the foregoing, it can be seen that transgenic plants expressing animal or fungal Δ -9 desaturase genes can be improved in their unsaturated 20 fatty acid composition by virtue of the activity of the foreign enzyme. Of further advantage, it has recently been discovered that some fungal Δ -9 desaturases (e.g., *Saccharomyces cerevisiae*) are fusion proteins comprising an intrinsic Cyt b₅ domain (Mitchell & Martin, J. Biol. 25 Chem. 270: 29766-29772, 1995). When this gene is expressed, sufficient Cyt b₅ is produced to drive the desaturase reaction at an optimum level and is not dependent on existing plant Cyt b₅. The known animal Δ -9 desaturases do not contain this fused Cyt b₅ motif and 30 must rely on independently-produced Cyt b₅ to provide the electrons for the reactions.

Though fungal or animal Δ -9 desaturases (e.g. the *S. cerevisiae* desaturase or the animal desaturases)

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may be expressed and functional in certain plants, their expression is likely less than optimal in plants, and expression may not even be possible in other plant species, due to several factors, including differences in 5 codon usage and codon preference in plants as compared to fungi, and among different plant species and the presence of cryptic intron splicing signals, among others. All of these factors can lead to poor expression, or no expression, of a non-plant foreign gene in a plant cell.

10 Accordingly, in order to make use of non-plant fatty acid desaturases, particularly those such as the *S. cerevisiae* Δ -9 desaturase comprising an internal Cyt b_s motif, a need exists to design modified desaturase-encoding DNA molecules that are customized for expression 15 in plant cells and specific plant tissues. It would be of even greater advantage to optimize such modified DNA molecules for expression in particular plant species, such as those that are grown and harvested primarily for oils.

20

SUMMARY OF THE INVENTION

According to one aspect of the invention, a synthetic fatty acid desaturase gene for expression in a multi-cellular plant is provided, the gene comprising a 25 desaturase domain and a Cyt b_s domain, wherein the gene is customized for expression in a plant cytoplasm. In one embodiment, the synthetic gene is customized for expression in a monocotyledonous plant. In another embodiment, the synthetic gene is customized for expression in a dicotyledonous plant. In a preferred 30 embodiment, the synthetic gene is customized for expression in a plant genus selected from the group consisting of *Arabidopsis*, *Brassica*, *Phaeolus*, *Oryza*,

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Olea, Elaeis (Oil Palm) and *Zea*.

In a preferred embodiment of the invention, the desaturase is a cytosolic Δ -9 desaturase. The *Saccharomyces cerevisiae* Δ -9 desaturase is particularly preferred.

In another embodiment of the invention, the synthetic gene is customized from a naturally occurring gene comprising both a desaturase domain and a cyt b_5 domain. Alternatively, the synthetic gene is a chimeric gene comprising a desaturase domain and a heterologous cyt b_5 domain.

In another embodiment, the synthetic gene is customized from a naturally occurring gene such that the synthetic gene and the naturally occurring gene encode an identical amino acid sequence. Alternatively, the synthetic gene is customized from a naturally occurring gene such that the synthetic gene and the naturally occurring gene encode a similar and functionally conserved amino acid sequence.

In another embodiment, a naturally occurring or a synthetic gene is customized so that specific amino acid modification are made to enhance the function of the encoded protein. Examples of such modifications include changing amino acids that are subjected to phosphorylation or other post-translational modifications that may alter or regulate the activity of the Δ -9 desaturase enzyme.

In another embodiment of the invention, elements of a naturally occurring or a synthetic desaturase gene that are not essential for enzymatic function are replaced or linked with elements derived from plant ER lipid biosynthetic genes that are normally expressed in maturing seeds or other plant tissues. The

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improved expression of the modified gene produced by the inclusion or substitution of plant DNA sequences in the synthetic gene will result from native plant signal or control elements in those sequences that affect

5 desaturase gene expression at one or more levels.

According to another aspect of the invention, a method is provided for constructing and customizing a bifunctional desaturase/cyt b₅ encoding gene for expression in the cytosol of a multicellular plant. The 10 method comprises (a) providing a DNA molecule comprising a desaturase-encoding moiety operably linked to a cyt b₅-encoding moiety, said DNA molecule producing the bifunctional polypeptide in a non-customized form; (b) back-translating the polypeptide sequence using preferred 15 codons for expression in a multicellular plant, thereby producing a back-translated nucleotide sequence; (c) analyzing the back-translated nucleotide sequence for features that could diminish or prevent expression in the plant cytoplasm, including, optionally (1) probable 20 intron splice sites (characterized by T-rich regions); (2) plant polyadenylation signals (e.g., AATAAA); (3) polymerase II termination sequence (e.g., CAN₍₇₋₉₎AGTNNAA, where N is any nucleotide); (4) hairpin consensus 25 sequences (e.g., UCUUCGG); and (5) the sequence-destabilizing motif ATTAA; (d) modifying the analyzed sequence to correct or remove the features that could diminish or prevent expression in the plant cytoplasm; and, optionally, (e) introducing desirable cloning 30 features, such as restriction sites, into the sequence in a manner that does not materially affect the desired codon usage or final polypeptide sequence.

The method set forth above may be adapted by incorporating into the customized gene one or more

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genomic segments from plant desaturase or other ER lipid biosynthetic genes, which are determined to further optimize gene expression in plants. This method comprises (1) identifying cDNA sequences that have 5 potential to comprise such beneficial elements, (2) creating yeast vectors expressing desaturase genes modified to contain these elements, (3) testing the vectors in a yeast expression system, (4) isolating regions from genomic DNA that are homologous to the 10 beneficial cDNA elements, and (6) using them to construct chimeric or hybrid synthetic genes that produce functional and highly efficient desaturase activities in plant tissues.

15 Other features and advantages of the present invention will be better understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

20 **Figure 1.** GCG Pileup comparison of stearoyl-CoA desaturase protein sequences. Sequences containing a Cyt b₅ domain are indicated with a +; sequences lacking a Cyt b₅ domain are indicated with a -; sequences still in question are indicated with a ?.

25 **Figure 2.** GCG Pileup comparison of Cytochrome b₅ protein sequences.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

30 Various terms relating to the biological molecules of the present invention are used herein above and also throughout the specifications and claims.

The term "promoter region" refers to the 5'

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regulatory regions of a gene.

The term "reporter gene" refers to genetic sequences which may be operably linked to a promoter region forming a transgene, such that expression of the reporter gene coding region is regulated by the promoter and expression of the transgene is readily assayed.

The term "selectable marker gene" refers to a gene product that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant.

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in an expression vector.

The term "DNA construct" refers to genetic sequence used to transform plants and generate progeny transgenic plants. These constructs may be administered to plants in a viral or plasmid vector. Other methods of delivery such as *Agrobacterium* T-DNA mediated transformation and transformation using the biolistic process are also contemplated to be within the scope of the present invention. The transforming DNA may be prepared according to standard protocols such as those set forth in "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 1999.

This invention provides synthetic DNA molecules (sometimes referred to herein as "synthetic genes") that

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encode a fatty acid desaturase useful for modifying the fatty acid composition of a plant. The DNA molecules describe in accordance with this invention are superior to DNA molecules currently available for this purpose, in 5 two important respects: (1) they encode a dual-domain polypeptide (sometimes referred to herein as a "bifunctional polypeptide or protein"), one domain being the fatty acid desaturase, and the other domain being cytochrome b_5 , a protein required to support the electron 10 transfer events that enable the desaturase to function; and (2) they are customized for expression in the cytosol of plant cells, and further customized for expression in particular selected plant species.

Design of synthetic genes of the present 15 invention is accomplished in two broad steps. First, the two components (the desaturase-encoding component and the Cyt b_5 -encoding component) are selected and linked together, if they do not occur together naturally. Second, the DNA molecule is optimized for expression in 20 the cytosol of a plant cell, or further for expression in a particular plant species, or group of species.

With regard to the first step, it should be noted that several fungal, animal and plant species, including yeast, are now known to contain naturally- 25 occurring genes encoding dual-domain cytoplasmic fatty acid desaturases. As mentioned above, the yeast and rat $\Delta-9$ desaturase genes have been expressed and shown to function in plants. However, prior to the present invention, it was not appreciated that the bifunctional yeast desaturase offers a significant advantage over the single-function animal desaturase in plant cells, where the requisite Cyt b_5 is available only in small amounts, and the yeast protein can provide its own supply of Cyt

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b₅.

With regard to the second step - optimization for expression in the plant cytosol - it was discovered in accordance with the present invention that a non-plant 5 desaturase-encoding gene, such as the yeast *OLE 1*, though expressed in some plants, may not be optimally expressed in those plants. Furthermore, the inventors have found that the yeast gene is poorly expressed in other plant species, thus highlighting the advantages obtainable by 10 optimizing such a gene for expression in a plant cell.

Sections II-IV below describe in detail how to design and use the synthetic genes of the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and 15 is not intended to limit the invention. Unless otherwise specified, general biochemical and molecular biological procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) 20 Current Protocols in Molecular Biology, John Wiley & Sons (1999) (hereinafter "Ausubel et al.") are used.

II. Design and construction of the synthetic DNA molecules

A. Selection of component DNA segments

25 This invention contemplates the use of the following source DNAs, which are thereafter modified for expression in plants, if necessary:

1. naturally occurring genes or cDNAs that encode dual domain polypeptides comprising a desaturase 30 domain and a Cyt b₅ domain;
2. chimeric genes in which a desaturase-encoding sequence from one source (e.g., the desaturase domain of a dual domain fungal Δ -9 desaturase, or the

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single domain rat desaturase), is linked to a Cyt b₅-encoding sequence from a different source (e.g., a plant);

3. chimeric genes in which a sequence that
5 encodes a fragment of a naturally occurring plant Cyt b₅ (e.g. the heme binding fold, or residues that comprise the electron donor or acceptor sites, or residues that act as membrane targeting or retention signals, or residues that act to stabilize the protein in the plant
10 cytoplasmic environment) is substituted for homologous regions in the cytochrome b₅ domain of a dual domain polypeptide such as the yeast Δ-9 desaturase; and

4. chimeric genes in which elements that encode the essential enzymatic domains from one source (e.g. a
15 native or synthetic gene derived from a fungal Δ-9 desaturase) are linked to elements derived from native plant desaturases that enhance transcription, mRNA processing, mRNA stability, protein folding and maturation, membrane targeting or retention, or protein
20 stability.

Naturally occurring genes or cDNAs that encode dual domain desaturase/Cyt b₅ proteins have been identified in several fungal species, including *Saccharomyces cerevisiae*, *Pichia augusta*, *Histoplasma capsulatum* and *Cryptococcus curvatus* (See Fig. 1).

Naturally occurring genes or cDNAs that encode independent, diffusible Cyt b₅ proteins have been identified in several plant species, including *Nicotiana tabacum* (tobacco), *Oryza sativa* (rice), *Cuscuta reflexa* 30 (*southern Asian dodder*), *Arabidopsis thaliana*, *Brassica oleracea* and *Olea europaea* (olive). A N-terminal Cyt b₅ domain of a Δ-6 desaturase has also been identified in the plant *Borago officinalis*, and in the *Saccharomyces*

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cerevisiae FAH1 gene that encodes a very long chain fatty acid hydroxylase. Genes or cDNAs from these species, as well as DNA from any other species identified in the future as encoding such a dual domain protein, are 5 contemplated for use in the synthetic genes of the present invention.

In a preferred embodiment, the yeast OLE1 gene is used. This embodiment is described in detail in Example 1.

10 The second strategy involves linking a DNA segment encoding a fatty acid desaturase from one source with a Cyt b₅ domain from another source. In a preferred embodiment, this chimeric gene is fashioned after the naturally-occurring dual function genes discussed above. 15 That is, the Cyt b₅ domain and the desaturase domain are situated in the same positions respective to each other as is found in the naturally occurring genes (see, e.g., Mitchell & Martin, J. Biol. Chem. 270: 29766-29772, 1996).

20 The chimeric dual-domain proteins of the invention are prepared by recombinant DNA methods, in which DNA sequences encoding each domain are operably linked together such that upon expression, a fusion protein having the desaturase and Cyt b₅ functions 25 described above is produced. As defined above, the term "operably linked" means that the DNA segments encoding the fusion protein are assembled with respect to each other, and with respect to an expression vector in which they are inserted, in such a manner that a functional 30 fusion protein is effectively expressed. The selection of appropriate promoters and other 5' and 3' regulatory regions, as well as the assembly of DNA segments to form an open reading frame, employs standard methodology well

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known to those skilled in the art.

Thus, preparing the chimeric DNAs of the invention involves selecting DNA sequences encoding each of the aforementioned components and operably linking the 5 respective sequences together in an appropriate vector. The sequences are thereafter expressed to produce the dual-function protein.

Genes or cDNAs that encode single-function cytoplasmic Δ -9 fatty acid desaturases have been 10 identified in a diverse array of prokaryotic and eucaryotic species, including insects, fungi and mammals, but not plants (Fig. 1). Genes or cDNAs from any of these species, as well as DNA from any other species identified in the future as encoding a fatty acid 15 desaturase, are contemplated for use in the synthetic genes of the present invention.

In preferred embodiments, desaturase-encoding genes from eucaryotes, most preferably fungi or mammals, are used. In a particularly preferred embodiment, a DNA 20 encoding the rat stearoyl CoA desaturase is used. This DNA has been successfully expressed in tobacco, and accordingly is expected to be useful as part of a chimeric desaturase/Cyt b₅ gene of the present invention.

Genes or cDNAs that encode Cyt b₅ proteins have 25 also been identified in a diverse array of eucaryotic species, including insects, fungi, mammals and plants. Genes or cDNAs from any of these species, as well as DNA from any other species identified in the future as encoding a Cyt b₅ protein, are contemplated for use in the 30 synthetic genes of the present invention.

In preferred embodiments, Cyt b₅-encoding genes or cDNAs from plants are used. These DNAs are preferred because they naturally comprise the codon usage preferred

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in plants, so require little, if any, of the modification steps described below for non-plant genes. Particularly preferred, if available, are Cyt b₅-encoding DNAs from the same plant species (or group of species) to be 5 transformed with the chimeric gene. For instance, synthetic chimeric genes constructed for transformation of *Brassica* species might comprise a stearoyl CoA-encoding domain from rat and a Cyt b₅ domain from *Brassica* (see Figs. 1 and 2 for specific sources). This chimeric 10 DNA would require optimization for expression in *Brassica* only in the desaturase domain.

With respect to the naturally-occurring dual domain-encoding genes, as well as the chimeric genes discussed above, it will be appreciated that the DNA 15 molecules can be prepared in a variety of ways, including DNA synthesis, cloning, mutagenesis, amplification, enzymatic digestion, and similar methods, all available in the standard literature. Additionally, certain DNA molecules can be obtained by access to public 20 repositories, such as the American Type Culture Collection. Alternatively, DNA molecules that are not readily available, and/or for which sequence information is not available, can be isolated from biological sources using standard hybridization methods and homologous 25 probes that are available.

B. Optimization for expression in plants

The second step in designing the synthetic DNA molecules of the invention is to customize (i.e. 30 optimize) their sequence for expression in the plant cytoplasm. This is accomplished by performing one or more of the steps listed below on the coding sequence of the above described non-plant (or chimeric)

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desaturase/Cyt b₅-encoding DNA molecules.

1. From the peptide sequence encoded by the DNA, back translate using an appropriate plant codon usage table, making certain in particular that the most 5 preferred translation termination codon is used.

2. Visually, or with the aid of computer software, analyze the back-translated nucleotide sequence for features that could diminish or prevent expression in the plant cytoplasm. Such features include: (1) probable 10 intron splice sites (characterized by T-rich regions); (2) plant polyadenylation signals (e.g., AATAAA); (3) polymerase II termination sequence (e.g., CAN₍₇₋₉₎AGTNNAA, where N is any nucleotide); (4) hairpin consensus sequences (e.g., UCUUCGG); and (5) the sequence- 15 destabilizing motif ATTTA (Shah & Kamen, Cell 46: 659-667, 1986). These features have been described in the art (U.S. Patent No., 5,500,365 to Fischhoff et al.; U.S. Patent No. 5,380,831 to Adang et al.).

3. Modify the back-translated sequence in 20 light of any "problem" sequences identified in step 2. Note that this step may require the introduction of codons that are not the most preferred, but instead are second or third-most preferred, in order to eliminate the more problematic sequences identified in step 2.

25 4. Introduce desirable cloning features, such as restriction sites, into the sequence in a manner that does not materially affect the desired codon usage or final polypeptide sequence.

The aforementioned optimization procedure can 30 be performed so that the final polypeptide sequence is identical to the initial polypeptide sequence, even though the underlying nucleotide sequence has been modified. This is a preferred embodiment of the

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invention. However, it is entirely feasible to modify the initial sequence such that the final sequence is not identical to the initial sequence, either by virtue of amino acid substitutions, insertions or deletions. The 5 more that is known about the structure/function relationship in a particular desaturase protein, the more liberties can be taken in modifying the protein sequence during the DNA optimization process. For instance, the present inventors have shown that the entire "coiled 10 coil" domain of the yeast *OLE1* gene can be deleted, and the protein remains functional. Thus, it appears that *OLE1* can tolerate significant modification in the encoded protein without losing its biological activity.

Codon usage tables for a variety of plants, 15 including general plant codon usage tables, tables for dicots, tables for monocots, and tables for particular species, are widely available. Some of these are reproduced in Example 1 below. One good location to access such tables is the website:

20

<http://biochem.octago.ac.nz.800/Transterm/codons.html>.

In an exemplary embodiment of the present invention, the above process is applied to the coding 25 sequence of the yeast *OLE1* gene, which encodes a cytoplasmically expressed dual-domain protein comprising a Δ-9 fatty acid desaturase domain and a Cyt b₅ domain. Optimization of the *OLE1* gene for expression in *Arabidopsis* and related species is described in detail in 30 Example 1.

In another preferred embodiment, the coding sequence of the rat stearoyl CoA desaturase is modified for expression in plants according to the methods

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described above. The modified sequence is operably linked to a coding sequence for a Cyt b₅ domain, preferably from a plant, and most preferably from *Brassica*. In this regard, it has been shown that 5 expression of this rat desaturase in tobacco produces a functional protein that increases the 16:1 fatty acid content of plant tissues. Splice site prediction analysis of the rat desaturase reveals that there are no plant intron-like sequences within the open reading 10 frame. However, codon usage analysis reveals that this desaturase possesses a number of codons that are not optimal for expression in plants, particularly *Arabidopsis* or *Brassica*.

In another preferred embodiment, the protein 15 coding sequences of the modified vectors described above are further modified to increase desaturase activity. This is done by altering specific amino acids in the encoded protein that control desaturase activity through post-translational modifications. These modifications 20 are presumed to increase the level of desaturase activity in the host plant by stabilizing the desaturase protein or by increasing catalytic activity of the desaturase. Post translational modifications such as protein phosphorylation or dephosphorylation have been shown to 25 alter activity of a number of enzymes by a number of different mechanisms. These include increasing or decreasing enzyme activity or protein stability, or changing the intracellular location of the enzyme. An examination of a wide range of Δ-9 desaturase enzymes 30 reveals the existence of a number of highly conserved potential phosphorylations sites that could serve as sequences that regulate desaturase activity. These are shown in bold face on the pile-up diagram in Figure 3 and

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are summarized in Table 1 of Example 3. The high degree of homology between these sites suggests that these sequences may also be recognized by host plant phosphorylating or dephosphorylating enzymes. If 5 phosphorylation of an amino acid within one of the sites increases the activity of the desaturase, the nucleic acid sequence corresponding to that amino acid can be altered to encode a negatively charged amino acid at that site to permanently increase the activity of the protein 10 in the host. If phosphorylation of an amino acid within the site reduces the activity of the desaturase enzyme, the nucleic acid sequence can be altered to replace that amino acid with a neutral amino acid that will permanently increase the activity of the enzyme.

15 In another preferred embodiment, elements of the genes in the modified vectors described above are further modified and improved by the linkage or substitution of sequences derived from native plant ER lipid biosynthetic genes. Those sequences contain 20 elements that improve the desaturase activity by increasing the efficiency of gene expression, intracellular protein targeting and/or enzyme stability. This is done by identifying elements of the engineered desaturase gene that can be replaced or linked with 25 elements of a plant gene without significantly affecting the desired activity or specificity of the resulting enzyme. Genes and cDNAs that encode ER lipid biosynthetic enzymes from *Brassica*, *Arabidopsis*, *Nicotiana tabacum*, *Borage*, *maize*, *sunflower* and *soybeans*, 30 as well as similar plant genes from any other species that are identified in the future, are contemplated for use in the synthetic genes of the present invention.

In connection with the aforementioned

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embodiment, but not limited thereto, it is particularly useful in many cases to pre-test constructs of the invention in a yeast expression system, in order to eliminate constructs that work poorly before taking the 5 more labor- and time-intensive step of testing them in plants. Accordingly, this step may be incorporated into the methods described herein.

10 **III. Construction of vectors for transforming plant nuclei, and production of transgenic plants expressing synthetic genes of the invention**

The synthetic genes of the present invention 15 are intended for use in producing transgenic plants that optimally express a dual-function desaturase/Cyt b₅ protein in the cytoplasm of plant cells. Transformation of plant nuclei to produce transgenic plants may be accomplished according to standard methods known in the 20 art. These include, but are not limited to, *Agrobacterium* vectors, PEG treatment of protoplasts, biolistic DNA delivery, UV laser microbeam, gemini virus vectors, calcium phosphate treatment of protoplasts, electroporation of isolated protoplasts, agitation of 25 cell suspensions with microbeads coated with the transforming DNA, direct DNA uptake, liposome-mediated DNA uptake, and the like. Such methods have been published in the art. See, e.g., Methods for Plant Molecular Biology, Weissbach & Weissbach eds., Academic 30 Press, Inc. (1988); Methods in Plant Molecular Biology, Schuler & Zielinski, eds., Academic Press, Inc. (1989); Plant Molecular Biology Manual, Gelvin Schilperoort, Verma, eds., Kluwer Academic Publishers, Dordrecht (1993); and Methods in Plant Molecular Biology - A 35 Laboratory Manual, Maliga, Klessig, Cashmore, Gruissem &

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Varner, eds., Cold Spring Harbor Press (1994).

The method of transformation depends upon the plant to be transformed. The biolistic DNA delivery method is useful for nuclear transformation, and is a 5 preferred method for practice of this invention. In another embodiment of the invention, *Agrobacterium* vectors are used to advantage for efficient transformation of plant nuclei.

In a preferred embodiment, the synthetic gene 10 is introduced into plant nuclei in *Agrobacterium* binary vectors. Such vectors include, but are not limited to, BIN19 (Bevan, Nucl. Acids Res., 12: 8711-8721, 1984) and derivatives thereof, the pBI vector series (Jefferson et al., EMBO J., 6: 3901-3907, 1987), and binary vectors 15 pGA482 and pGA492 (An, Plant Physiol., 81: 86-91, 1986). A new series of *Agrobacterium* binary vectors, the pPZP family, is preferred for practice of the present 20 invention. The use of this vector family for plant transformation is described by Svab et al. in Methods in Plant Molecular Biology - A Laboratory Manual, Maliga, Klessig, Cashmore, Gruissem and Varner, eds., Cold Spring Harbor Press (1994).

Using an *Agrobacterium* binary vector system for transformation, the synthetic gene of the invention is 25 linked to a nuclear drug resistance marker, such as kanamycin or gentamycin resistance. *Agrobacterium*-mediated transformation of plant nuclei is accomplished according to the following procedure:

- (1) the gene is inserted into the selected 30 *Agrobacterium* binary vector;
- (2) transformation is accomplished by co-cultivation of plant tissue (e.g., leaf discs) with a suspension of recombinant *Agrobacterium*, followed by

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incubation (e.g., two days) on growth medium in the absence of the drug used as the selective medium (see, e.g., Horsch et al., *Science* 227: 1229-1231, 1985);

5 (3) plant tissue is then transferred onto the selective medium to identify transformed tissue; and

(4) identified transformants are regenerated to intact plants.

It should be recognized that the amount of expression, as well as the tissue specificity of 10 expression of the synthetic genes in transformed plants can vary depending on the position of their insertion into the nuclear genome. Such position effects are well known in the art; see Weising et al., *Ann. Rev. Genet.*, 22: 421-477 (1988). For this reason, several nuclear 15 transformants should be regenerated and tested for expression of the synthetic gene.

IV. Uses of the synthetic genes and transgenic plants expressing those genes

20 The synthetic desaturase genes of the invention and transgenic plants expressing those genes can be used for several agriculturally beneficial purposes. For instance, they can be used in oil-producing crops (e.g., 25 corn, soybean, sunflower, rapeseed) to increase the overall percentages of monounsaturated fatty acids in those oils, thereby improving their health-promoting qualities. In this regard, the production of transgenic rapeseed plants (*Brassica napus*) is of particular 30 interest in this invention. Example 1 describes a synthetic yeast desaturase gene modified for expression in *Arabidopsis*. Because the codon usage of *Brassica* is very similar to that of *Arabidopsis*, it is expected that the synthetic gene described in Example 1 will be as well

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expressed in *Brassica* as it is in *Arabidopsis*.

Another use for the synthetic genes of the invention is to modify the flavors of certain fruit or vegetable crops. It has already been shown that 5 expression of the un-modified yeast Δ -9 desaturase gene in tomato results in alterations in fatty acid composition and fatty acid-derived flavor compounds (Wang et al., 1996, *supra*). The synthetic, plant-optimized version of this gene is expected to function similarly, 10 and also to be more efficiently expressed in plant cells.

Another use for the synthetic genes of the invention is to facilitate the formation of omega-5 anacardic acids, a class of secondary compounds derived from the Δ -9 desaturation of 14:0 in pest-resistant 15 geraniums (Schultz et al., Proc. Natl. Acad. Sci. USA, 93: 877-885, 1996). It has been shown that formation of these compounds proceeds from the expression of Δ 9 desaturase activity resulting in the formation of Δ 9 14:1. Subsequent elongation of these molecules leads to 20 the formation of omega-5 22:1 and 24:1 in the trichome exudate that leads to pest resistance against spider mites and aphids.

Another use for the synthetic genes of the invention are in the modification of membrane lipid fatty 25 acyl composition to alter the properties of the cytoplasmic and plasma membranes of the cell. These may affect functions such membrane associated activities that are associated with membrane functions such as signal transduction, endocytosis or exocytotic events, entry of 30 fungal or viral pathogens into the cell, and temperature or environmentally caused stress that causes physical changes in the fluid properties of the plasma membrane or internal cell membranes. Plants defective in desaturases

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have been reported (Somerville and Browse, *supra*). These mutant plants contain higher than normal levels of saturated fatty acids that may lower membrane fluidity under normal growing conditions. Thus the effects of 5 temperature on these plants involved high temperature tolerance as opposed to chilling tolerance. These studies yielded interesting information that has relevance to temperature stress in general. A mutant of *Arabidopsis* deficient in 16:0 desaturation (Hugly et al, 10 *Plant Physiol.* 90: 1134-1142) for example, has been shown to appear and grow normally at non-stressful temperatures. Under high temperature conditions, however, the mutant performs better than controls in growth and biosynthetic studies. Higher temperature 15 stability was also noted in pea thylakoids following catalytic hydrogenation (Thoman et al. *Biochem. Biophys. Acta* 849: 131-140, 1986).

20 The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

EXAMPLE 1

25 **Modification of the *Saccharomyces cerevisiae OLE1 Gene for Expression in *Arabidopsis* and Related Species***

When introduced into tobacco and tomato plants, the yeast Δ -9 desaturase gene (*OLE1*) was shown to 30 desaturate palmitate and stearate, thereby reducing the levels of saturated fatty acids in triglycerides (Polashock et al., *supra*; Wang et al., *supra*). However, it was unclear whether optimum expression of the *OLE1* gene occurred in those species, and expression in other

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plant species has been less than optimum. For example, the present inventors have found that the level of expression of the *OLE1* gene in tobacco (Polashock, et al., *Plant Physiol.* 100:894-901, 1992) and *Arabidopsis*

5 varies in different plant tissues and is generally poor in tobacco, and *Arabidopsis* seeds. Similarly, data from other investigators indicate that expression of *OLE1* in rapeseed (*Brassica napus*) seeds is also poor (U.S. Patent No. 5,777,201, to Poutre, et al.).

10 Differential expression of heterologous genes in plants can be caused by several factors. It is often due to the presence of cryptic intron splicing signals. Thus, it is possible that the multiple banding patterns observed in northern blots of *OLE1*-transformed tobacco
15 (Polashock et al., *supra*) are due to splicing of the *OLE1* mRNA.

20 In plants, the mRNA splicing mechanism is less well defined than in mammalian or yeast systems. There is some conservation of the 5' and 3' splicing signals but there is no conserved internal splice signal.

25 However, with the accumulation of plant genomic DNA sequence data, it is now becoming possible to predict with some accuracy where intron splicing will occur (Hebsgaard, S.M., P.G. Korning, N. Tolstrup, J.

Engelbrecht, P. Rouze and S. Brunak, *Nucleic Acids Research* 24(17): 3439-3452, 1996). In fact, computer programs that predict splice sites have now been developed (the "PlantNetGene" server for splice site predictions: <http://www.cbs.dtu.dk/NetPlantGene.html>).

30 From these sources, it appears that plant introns are typically identified as T rich sequences.

Another factor affecting expression of foreign genes in plants is codon preference. It is now well

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known that preference for certain codons exist among different phyla, classes, families, genera and species. Accordingly, by modifying a DNA sequence so that it uses codons preferred in a particular organism, expression of 5 that sequence can be optimized.

Other factors affecting the expression of foreign genes in plants include the presence of putative polyadenylation signals, hairpin cleavage consensus motifs, polymerase II termination sequences and the Shaw-10 Kamen sequence pattern ATTTA.

This example describes the design and construction of "pl-ole1", a modified *Saccharomyces cerevisiae OLE1* gene optimized for expression in *Arabidopsis* and other plant species.

15 The nucleotide sequence of the *Saccharomyces cerevisiae OLE1* gene coding sequence has been described in U.S. Patent No. 5,057,419 to Martin et al. (incorporated by reference herein) and is set forth below for convenience as SEQ ID NO:1 (open reading frame starts 20 at +11). The *S. cerevisiae* Δ-9 desaturase amino acid sequence encoded by *OLE1* is set forth as SEQ ID NO:2.

I. Design of pl-ole1

To modify *OLE1* for optimum expression in plants, the *OLE1* sequence was first analyzed for cryptic 25 plant splice signals, using the PlantNetGene server for splice site predictions. This analysis identified a number of "high confidence" intron splice signals in the *OLE1* sequence. These are shown below (positions correspond to position numbers in SEQ ID NO:1).

30 Donor splice site, direct strand:

<u>Position</u>	<u>Strand</u>	<u>Confidence</u>	<u>exon^intron</u>
5' - 3'			5' - 3'
(Start ATG = +1)			

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397	+	1.00	GCTCTCTCTG [^] GTAAAGTACC
1052	+	0.85	CTATTAAGTG [^] GTACCAATAC
1074	+	1.00	CCCAACTAAG [^] GTTATCATCT

Acceptor splice site, direct strand:

5	5' - 3'		5' - 3'
	<u>Position</u>	<u>Strand</u>	<u>Confidence</u>
			<u>intron[^]exon</u>
	500	+	0.86 GGTCTCACAG [^] ATCTTACTCC

10 Next, the *OLE1* peptide sequence (SEQ ID NO:2) was back-translated using an *Arabidopsis thaliana* codon usage table, as shown below. Codon usage in *Arabidopsis* and several other plant species, including *Brassica napus*, *Phaseolus vulgaris* and *Zea mays* is very similar, 15 as can be seen by a comparison with the respective codon usage tables of those species, also shown below (the codon usage table of *Saccharomyces cerevisiae* is shown for comparison; codon usage tables taken from [Ahttp://biochem.otago.ac.nz:800/ Transterm/codons.html](http://biochem.otago.ac.nz:800/Transterm/codons.html)).

20

Arabidopsis thaliana.

	AmAcid	Codon	Number	/1000	Fraction	..
25	Gly	GGG	6027.00	10.31	0.14	
	Gly	GGA	15393.00	26.32	0.37	
	Gly	GGT	14890.00	25.46	0.35	
	Gly	GGC	5654.00	9.67	0.13	
30	Glu	GAG	19825.00	33.90	0.51	
	Glu	GAA	18672.00	31.93	0.49	
	Asp	GAT	20862.00	35.67	0.65	
	Asp	GAC	11061.00	18.91	0.35	
35	Val	GTG	10414.00	17.81	0.26	
	Val	GTA	5145.00	8.80	0.13	
	Val	GTT	16157.00	27.63	0.41	
	Val	GTC	8156.00	13.95	0.20	
40	Ala	GCG	5361.00	9.17	0.13	
	Ala	GCA	10552.00	18.04	0.25	
	Ala	GCT	18782.00	32.12	0.45	

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	Ala	GCC	7249.00	12.40	0.17
5	Arg	AGG	6684.00	11.43	0.22
	Arg	AGA	10280.00	17.58	0.34
	Ser	AGT	7369.00	12.60	0.16
	Ser	AGC	6399.00	10.94	0.14
10	Lys	AAG	20436.00	34.94	0.55
	Lys	AAA	16882.00	28.87	0.45
	Asn	AAT	11658.00	19.93	0.47
	Asn	AAC	12987.00	22.21	0.53
15	Met	ATG	14817.00	25.34	1.00
	Ile	ATA	6571.00	11.24	0.21
	Ile	ATT	13028.00	22.28	0.41
	Ile	ATC	11855.00	20.27	0.38
20	Thr	ACG	4346.00	7.43	0.14
	Thr	ACA	8703.00	14.88	0.28
	Thr	ACT	10909.00	18.65	0.36
	Thr	ACC	6720.00	11.49	0.22
25	Trp	TGG	6868.00	11.74	1.00
	End	TGA	652.00	1.11	0.44
	Cys	TGT	5641.00	9.65	0.58
	Cys	TGC	4154.00	7.10	0.42
30	End	TAG	252.00	0.43	0.17
	End	TAA	591.00	1.01	0.40
	Tyr	TAT	8052.00	13.77	0.47
	Tyr	TAC	8965.00	15.33	0.53
35	Leu	TTG	11727.00	20.05	0.22
	Leu	TTA	6361.00	10.88	0.12
	Phe	TTT	11703.00	20.01	0.47
	Phe	TTC	13066.00	22.34	0.53
40	Ser	TCG	4830.00	8.26	0.10
	Ser	TCA	9033.00	15.45	0.19
	Ser	TCT	13022.00	22.27	0.28
	Ser	TCC	6214.00	10.63	0.13
45	Arg	CGG	2531.00	4.33	0.08
	Arg	CGA	3142.00	5.37	0.10
	Arg	CGT	5680.00	9.71	0.19
	Arg	CGC	2100.00	3.59	0.07
	Gln	CAG	9564.00	16.35	0.47
	Gln	CAA	10908.00	18.65	0.53

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	His	CAT	7466.00	12.77	0.58
	His	CAC	5415.00	9.26	0.42
5	Leu	CTG	5669.00	9.69	0.11
	Leu	CTA	5350.00	9.15	0.10
	Leu	CTT	14395.00	24.61	0.27
	Leu	CTC	9751.00	16.67	0.18
10	Pro	CCG	4676.00	8.00	0.17
	Pro	CCA	9131.00	15.61	0.33
	Pro	CCT	10732.00	18.35	0.39
	Pro	CCC	3331.00	5.70	0.12

15 *Brassica napus*

	AmAcid	Codon	Number	/1000	Fraction ..
20	Gly	GGG	730.00	11.21	0.13
	Gly	GGA	2042.00	31.37	0.36
	Gly	GGT	1952.00	29.99	0.35
	Gly	GGC	892.00	13.70	0.16
25	Glu	GAG	2119.00	32.55	0.55
	Glu	GAA	1764.00	27.10	0.45
	Asp	GAT	1895.00	29.11	0.56
	Asp	GAC	1478.00	22.70	0.44
30	Val	GTG	1231.00	18.91	0.28
	Val	GTA	493.00	7.57	0.11
	Val	GTT	1624.00	24.95	0.36
	Val	GTC	1124.00	17.27	0.25
35	Ala	GCG	615.00	9.45	0.13
	Ala	GCA	1167.00	17.93	0.24
	Ala	GCT	2028.00	31.15	0.42
	Ala	GCC	1056.00	16.22	0.22
40	Arg	AGG	697.00	10.71	0.22
	Arg	AGA	996.00	15.30	0.32
	Ser	AGT	736.00	11.31	0.15
	Ser	AGC	803.00	12.34	0.17
45	Lys	AAG	2243.00	34.46	0.55
	Lys	AAA	1817.00	27.91	0.45
	Asn	AAT	1058.00	16.25	0.37
	Asn	AAC	1811.00	27.82	0.63
	Met	ATG	1538.00	23.63	1.00

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	Ile	ATA	669.00	10.28	0.20
	Ile	ATT	1271.00	19.52	0.37
	Ile	ATC	1461.00	22.44	0.43
5	Thr	ACG	563.00	8.65	0.15
	Thr	ACA	1059.00	16.27	0.28
	Thr	ACT	1154.00	17.73	0.30
	Thr	ACC	1073.00	16.48	0.28
10	Trp	TGG	798.00	12.26	1.00
	End	TGA	69.00	1.06	0.37
	Cys	TGT	517.00	7.94	0.50
	Cys	TGC	509.00	7.82	0.50
15	End	TAG	33.00	0.51	0.18
	End	TAA	83.00	1.28	0.45
	Tyr	TAT	792.00	12.17	0.38
	Tyr	TAC	1283.00	19.71	0.62
20	Leu	TTG	1051.00	16.14	0.20
	Leu	TTA	508.00	7.80	0.09
	Phe	TTT	1003.00	15.41	0.39
	Phe	TTC	1562.00	23.99	0.61
25	Ser	TCG	475.00	7.30	0.10
	Ser	TCA	856.00	13.15	0.18
	Ser	TCT	1147.00	17.62	0.24
	Ser	TCC	799.00	12.27	0.17
	Arg	CGG	219.00	3.36	0.07
	Arg	CGA	297.00	4.56	0.09
	Arg	CGT	659.00	10.12	0.21
30	Arg	CGC	275.00	4.22	0.09
	Gln	CAG	1188.00	18.25	0.50
	Gln	CAA	1168.00	17.94	0.50
	His	CAT	651.00	10.00	0.49
35	His	CAC	672.00	10.32	0.51
	Leu	CTG	592.00	9.09	0.11
	Leu	CTA	579.00	8.89	0.11
	Leu	CTT	1416.00	21.75	0.26
	Leu	CTC	1208.00	18.56	0.23
40	Pro	CCG	542.00	8.33	0.15
	Pro	CCA	1180.00	18.13	0.33
	Pro	CCT	1281.00	19.68	0.36
	Pro	CCC	527.00	8.10	0.15

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Phaseolus vulgaris

	Gly	GGG	371.00	13.30	0.15
	Gly	GGA	771.00	27.64	0.32
5	Gly	GGT	817.00	29.29	0.34
	Gly	GGC	441.00	15.81	0.18
	Glu	GAG	912.00	32.69	0.54
	Glu	GAA	767.00	27.50	0.46
10	Asp	GAT	776.00	27.82	0.55
	Asp	GAC	625.00	22.41	0.45
	Val	GTG	661.00	23.70	0.36
	Val	GTA	174.00	6.24	0.09
15	Val	GTT	653.00	23.41	0.36
	Val	GTC	346.00	12.40	0.19
	Ala	GCG	180.00	6.45	0.09
	Ala	GCA	528.00	18.93	0.26
20	Ala	GCT	791.00	28.36	0.39
	Ala	GCC	553.00	19.82	0.27
	Arg	AGG	324.00	11.61	0.29
	Arg	AGA	325.00	11.65	0.29
25	Ser	AGT	317.00	11.36	0.14
	Ser	AGC	353.00	12.65	0.15
	Lys	AAG	1054.00	37.78	0.60
	Lys	AAA	697.00	24.99	0.40
30	Asn	AAT	555.00	19.90	0.42
	Asn	AAC	782.00	28.03	0.58
	Met	ATG	567.00	20.33	1.00
	Ile	ATA	274.00	9.82	0.20
35	Ile	ATT	539.00	19.32	0.40
	Ile	ATC	548.00	19.65	0.40
	Thr	ACG	166.00	5.95	0.11
	Thr	ACA	362.00	12.98	0.24
40	Thr	ACT	480.00	17.21	0.32
	Thr	ACC	490.00	17.57	0.33
	Trp	TGG	342.00	12.26	1.00
	End	TGA	34.00	1.22	0.44
45	Cys	TGT	145.00	5.20	0.39
	Cys	TGC	229.00	8.21	0.61
	End	TAG	22.00	0.79	0.28
	End	TAA	22.00	0.79	0.28

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	Tyr	TAT	400.00	14.34	0.40
	Tyr	TAC	597.00	21.40	0.60
5	Leu	TTG	543.00	19.47	0.24
	Leu	TTA	184.00	6.60	0.08
	Phe	TTT	458.00	16.42	0.43
	Phe	TTC	601.00	21.55	0.57
10	Ser	TCG	149.00	5.34	0.06
	Ser	TCA	416.00	14.91	0.18
	Ser	TCT	606.00	21.72	0.26
	Ser	TCC	501.00	17.96	0.21
15	Arg	CGG	71.00	2.55	0.06
	Arg	CGA	76.00	2.72	0.07
	Arg	CGT	169.00	6.06	0.15
	Arg	CGC	158.00	5.66	0.14
20	Gln	CAG	437.00	15.67	0.48
	Gln	CAA	470.00	16.85	0.52
	His	CAT	298.00	10.68	0.46
	His	CAC	355.00	12.73	0.54
25	Leu	CTG	351.00	12.58	0.15
	Leu	CTA	184.00	6.60	0.08
	Leu	CTT	569.00	20.40	0.25
	Leu	CTC	452.00	16.20	0.20
30	Pro	CCG	147.00	5.27	0.08
	Pro	CCA	694.00	24.88	0.37
	Pro	CCT	664.00	23.80	0.36
	Pro	CCC	352.00	12.62	0.19

35 **Zea mays**

	AmAcid	Codon	Number	/1000	Fraction	..
40	Gly	GGG	2466.00	15.07	0.19	
	Gly	GGA	2186.00	13.36	0.17	
	Gly	GGT	2607.00	15.93	0.20	
	Gly	GGC	5499.00	33.61	0.43	
45	Glu	GAG	7364.00	45.01	0.72	
	Glu	GAA	2823.00	17.25	0.28	
	Asp	GAT	3425.00	20.93	0.37	
	Asp	GAC	5740.00	35.08	0.63	
	Val	GTG	4365.00	26.68	0.38	

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	Val	GTA	916.00	5.60	0.08
	Val	GTT	2516.00	15.38	0.22
	Val	GTC	3644.00	22.27	0.32
5	Ala	GCG	3698.00	22.60	0.24
	Ala	GCA	2517.00	15.38	0.16
	Ala	GCT	3602.00	22.01	0.24
	Ala	GCC	5481.00	33.50	0.36
10	Arg	AGG	2500.00	15.28	0.27
	Arg	AGA	1199.00	7.33	0.13
	Ser	AGT	1170.00	7.15	0.10
	Ser	AGC	2776.00	16.97	0.24
15	Lys	AAG	7241.00	44.25	0.79
	Lys	AAA	1969.00	12.03	0.21
	Asn	AAT	1946.00	11.89	0.33
	Asn	AAC	3939.00	24.07	0.67
20	Met	ATG	4071.00	24.88	1.00
	Ile	ATA	1014.00	6.20	0.13
	Ile	ATT	2099.00	12.83	0.28
	Ile	ATC	4403.00	26.91	0.59
25	Thr	ACG	1890.00	11.55	0.22
	Thr	ACA	1620.00	9.90	0.19
	Thr	ACT	1757.00	10.74	0.21
	Thr	ACC	3236.00	19.78	0.38
30	Trp	TGG	1994.00	12.19	1.00
	End	TGA	199.00	1.22	0.45
	Cys	TGT	770.00	4.71	0.28
	Cys	TGC	1963.00	12.00	0.72
35	End	TAG	121.00	0.74	0.28
	End	TAA	120.00	0.73	0.27
	Tyr	TAT	1303.00	7.96	0.27
	Tyr	TAC	3440.00	21.02	0.73
40	Leu	TTG	1807.00	11.04	0.13
	Leu	TTA	582.00	3.56	0.04
	Phe	TTT	1697.00	10.37	0.29
	Phe	TTC	4082.00	24.95	0.71
45	Ser	TCG	1620.00	9.90	0.14
	Ser	TCA	1592.00	9.73	0.14
	Ser	TCT	1792.00	10.95	0.15
	Ser	TCC	2746.00	16.78	0.23

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	Arg	CGG	1505.00	9.20	0.16
	Arg	CGA	610.00	3.73	0.06
	Arg	CGT	1018.00	6.22	0.11
	Arg	CGC	2562.00	15.66	0.27
5					
	Gln	CAG	4280.00	26.16	0.72
	Gln	CAA	1626.00	9.94	0.28
	His	CAT	1378.00	8.42	0.36
	His	CAC	2431.00	14.86	0.64
10					
	Leu	CTG	4069.00	24.87	0.29
	Leu	CTA	904.00	5.52	0.07
	Leu	CTT	2415.00	14.76	0.17
	Leu	CTC	4079.00	24.93	0.29
15					
	Pro	CCG	2642.00	16.15	0.29
	Pro	CCA	2152.00	13.15	0.23
	Pro	CCT	2102.00	12.85	0.23
	Pro	CCC	2344.00	14.33	0.25
20					

Saccharomyces cerevisiae

	AmAcid	Codon	Number	/1000	Fraction	..
25	Gly	GGG	18129.00	6.18	0.12	
	Gly	GGA	32850.00	11.20	0.22	
	Gly	GGT	66575.00	22.69	0.45	
	Gly	GGC	28821.00	9.82	0.20	
30	Glu	GAG	57100.00	19.46	0.30	
	Glu	GAA	133513.00	45.51	0.70	
	Asp	GAT	111120.00	37.88	0.65	
	Asp	GAC	58642.00	19.99	0.35	
35	Val	GTG	32144.00	10.96	0.20	
	Val	GTA	35470.00	12.09	0.22	
	Val	GTT	63678.00	21.71	0.39	
	Val	GTC	33136.00	11.30	0.20	
40	Ala	GCG	18402.00	6.27	0.11	
	Ala	GCA	47728.00	16.27	0.30	
	Ala	GCT	58916.00	20.08	0.37	
	Ala	GCC	35917.00	12.24	0.22	
45	Arg	AGG	27990.00	9.54	0.21	
	Arg	AGA	61524.00	20.97	0.47	
	Ser	AGT	42499.00	14.49	0.16	
	Ser	AGC	29298.00	9.99	0.11	

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	Lys	AAG	89539.00	30.52	0.42
	Lys	AAA	124327.00	42.38	0.58
	Asn	AAT	106379.00	36.26	0.60
	Asn	AAC	71659.00	24.43	0.40
5					
	Met	ATG	61216.00	20.87	1.00
	Ile	ATA	53773.00	18.33	0.28
	Ile	ATT	88869.00	30.29	0.46
	Ile	ATC	49422.00	16.85	0.26
10					
	Thr	ACG	24131.00	8.23	0.14
	Thr	ACA	52363.00	17.85	0.31
	Thr	ACT	58260.00	19.86	0.34
	Thr	ACC	35998.00	12.27	0.21
15					
	Trp	TGG	30707.00	10.47	1.00
	End	TGA	1901.00	0.65	0.30
	Cys	TGT	23942.00	8.16	0.62
	Cys	TGC	14448.00	4.93	0.38
20					
	End	TAG	1421.00	0.48	0.23
	End	TAA	2985.00	1.02	0.47
	Tyr	TAT	55441.00	18.90	0.57
	Tyr	TAC	42016.00	14.32	0.43
25					
	Leu	TTG	79248.00	27.01	0.28
	Leu	TTA	77691.00	26.48	0.28
	Phe	TTT	78451.00	26.74	0.59
	Phe	TTC	53809.00	18.34	0.41
30					
	Ser	TCG	25856.00	8.81	0.10
	Ser	TCA	55962.00	19.08	0.21
	Ser	TCT	69019.00	23.53	0.26
	Ser	TCC	41460.00	14.13	0.16
35					
	Arg	CGG	5414.00	1.85	0.04
	Arg	CGA	9166.00	3.12	0.07
	Arg	CGT	18429.00	6.28	0.14
	Arg	CGC	7924.00	2.70	0.06
40					
	Gln	CAG	36018.00	12.28	0.31
	Gln	CAA	78385.00	26.72	0.69
	His	CAT	40211.00	13.71	0.64
	His	CAC	22609.00	7.71	0.36
45					
	Leu	CTG	31503.00	10.74	0.11
	Leu	CTA	39789.00	13.56	0.14
	Leu	CTT	36697.00	12.51	0.13
	Leu	CTC	16401.00	5.59	0.06

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Pro	CCG	15796.00	5.38	0.12
Pro	CCA	51725.00	17.63	0.41
Pro	CCT	39402.00	13.43	0.31
Pro	CCC	20387.00	6.95	0.16

5

For each amino acid, the new *pl-ole1* gene was designed the codon most preferred in *Arabidopsis*, with the following exceptions:

10 1. The codon for glutamine CAG was switched to CAA. Though the codon preference for glutamine is the same for both CAG and CAA in *Arabidopsis*, CAA was used since the AG motif is part of the 3' intron splice signal.

15 2. In *OLE1*, there are regions of high leucine/valine amino acid usage (e.g., between positions 322 to 571 of the nucleotide sequence are codons coding for 11 leucines and 7 valines). These regions correspond to the *OLE1* protein transmembrane domains. If the most 20 preferred codons in *Arabidopsis* (CTT and GTT, respectively) were used, the region would take on the characteristics of a plant intron, i.e., high T content, thereby introducing a number of highly probable 5' splice sites, which could not be removed without altering the 25 amino acid sequence. Accordingly, a mixture of alternative codons was used for these amino acids. Similar changes were also applied to two other regions of *OLE1* (positions 781 to 900 and positions 1081 to 1140).

Next, a search for problematic sequences, such 30 as putative polyadenylation signals, hairpin cleavage consensus motifs, ATTTA motifs or concatamers thereof, was conducted. Such sequences are described in detail in U.S. Patent No. 5,380,831 to Adang et al. (incorporated by reference herein). This search identified one hairpin 35 cleavage consensus motif, CTTCGG, at position 553-559 of

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SEQ ID NO:1, which was removed by changing TTC to TTT (both encoding phenylalanine).

Next, a BamHI site and translation initiation consensus were added to the 5' end of the *OLE1* coding sequence (M. Kozak, J. Biol. Chem. 266(30): 19867-19870, 1991). An XbaI and a BamHI site were added to the 3' end of the coding sequence. A PacI site was introduced into the same position as the original *S. cerevisiae* *OLE1* PacI site (within the cytochrome b₅ domain), in order to provide a convenient restriction site for construction of this and other synthetic *OLE1* genes. Other convenient restriction sites, which enable modular construction of synthetic *OLE1* genes, are inherent within the final sequence of the new *pl-ole1* gene.

Finally, the termination codon was checked against a stop codon consensus database, "TransTerm" (Dolphin et al., Nucl. Acids Res. 25(1): 246-247, 1997). The existing termination sequence, TGAT, appeared suitable for use in *Arabidopsis*, and so was not altered.

20 **II. Construction of pl-ole1:**

The rebuilt *pl-ole1* nucleotide sequence was constructed commercially (Operon Technologies, Inc.). The plasmid containing the rebuilt gene was designated pAMCM013. The *pl-ole1* nucleotide sequence is set forth below as SEQ ID NO:3 (open reading frame starts at +11). This sequence encodes SEQ ID NO:2, but differs from the *S. cerevisiae* *OLE1* gene (SEQ ID NO:1) in the following respects (summarized from above):

1. *Arabidopsis thaliana* codon usage; CAG 30 switched to CAA for glutamine;
2. Translation initiation consensus added;
3. Hairpin removed;
4. Several (but not all) PlantNetGene

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predicted splice sites removed;

5. Eleven leucines changed from CTT to CTC, and 7 valines changed from GTT to GTG in positions 322-571, which corresponds to a plant intron-like region; similar changes made in regions 781-900 and 1081-1140; valine at position 432 retained as GTT to maintain Psp1406I site;

6. Certain leucine and valine codons were altered so that the same codons would not appear adjacent to others;

7. Intron acceptor site at position 1047
altered;

8. Restriction sites added to allow modular construction; PsP1406I site removed at position 1441; and

15 9. PacI site introduced at position 1362; an introduced NgoMI site at position 867 removed.

A gap alignment of SEQ ID NO: 1 (top) and SEQ ID NO: 3 (bottom) is shown below:

Gap alignment of wild type and rebuilt OLE1 sequences.
Percent Similarity: 79.871 Percent Identity: 79.871

1 TACAACAAAGATGCCAACCTCTGGAACTACTATTGAATTGATTGACGACC 50
1 ggatccaacaATGCCTACTTCTGGAACTACTATCGAGCTTATCGATGATC 50
51 AATTTCCTAAAGGATGACTCTGCCAGCAGTGGCATTGTCGACGAAGTCGAC 100
51 AATTCCCTAAGGATGATTCTGCTTCTTCTGGAAATCGTTGATGAGGTTGAT 100
101 TTAACGGAAGCTAATATTTGGCTACTGGTTGAATAAGAAAGCACCAAG 150
101 CTTACTGAGGCTAACATCCTGCTACTGGACTTAACAAGAAGGCTCCTAG 150
151 AATTGTCAACGGTTGGTTCTTAAATGGGCTCCAAGGAAATGGTTCCG 200
151 AATCGTTAACGGATTGGATCTCTTATGGGATCTAAGGAGATGGTTCTG 200
201 TGGAATTGACAAGAAGGGAAACGAAAAGAAGTCCAATTGGATCGTCTG 250
201 TTGAGTTGATAAGAAGGGAAACGAGAAGAAGTCTAACCTTGATAGACTT 250

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251 CTAGAAAAGGACAACCAAGAAAAAGAAGAAGCTAAAACATAAAATTACAT 300
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
251 CTTGAGAAGGATAACCAAGAGAAGGAGGAGGCTAACAGACTAACATCCATAT 300
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
301 CTCCGAACAACCATGGACTTGAATAACTGGCACCAACATTGAACGGT 350
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
301 CTCTGAGCAACCTGGACTCTcAACAACTGGCATCAACATCTcAACTGGC 350
||| ||| ||| ||| ||| ||| ||| |||
351 TGAACATGGTCTTGTGTTGGTATGCCAATGATTGGTTGGTACTTCGCT 400
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
351 TcAACATGGTgCTcGTcTGTGGAATGCCTATGATCGGATGGTACTTCGCT 400
||| ||| ||| ||| ||| ||| ||| |||
401 CTCTCTGGTAAAGTACCTTGCATTTAACGTTTCTTTCTCCGTTT 450
||| ||| ||| ||| ||| ||| ||| ||| |||
401 CTcTCTGGAAAaGTgCCTCTcCATCTcAACGTTTCCtCtTTCTCTGTcTT 450
||| ||| ||| ||| ||| ||| |||
451 CTACTACGCTGTCGGTGGTGTCTATTACTGCCGTTACCATAGATTAT 500
||| ||| ||| ||| ||| ||| ||| |||
451 CTACTACGCTGTTGGAGGGAGTgTCTATCACTGCTGGATACCATAGACTcT 500
||| ||| ||| ||| ||| ||| |||
501 GGTCTCACAGATCTTACTCCGCTCACTGGCCATTGAGATTATTCTACGCT 550
||| ||| ||| ||| ||| ||| |||
501 GGTCTCATAGATCTTACTCTGCTCATGGCCTCTAGACTcTTCTACGCT 550
||| ||| ||| ||| ||| |||
551 ATCTTCGGTTGTGCTTCCGTTGAAGGGCCGCTAAATGGTGGGCCACTC 600
||| ||| ||| ||| ||| ||| ||| |||
551 ATCTTtGGATGTGCTTCTGTTGAGGGATCTGCTAAGTGGTGGGCCATTC 600
||| ||| ||| ||| ||| |||
601 TCACAGAATTCAACCATCGTTACACTGATACCTTGAGAGATCCTTATGACG 650
||| ||| ||| ||| ||| ||| |||
601 TCATAGAATCCATCATAGATACTGATACTCTTAGAGATCCTACGATG 650
||| ||| ||| ||| |||
651 CTCGTAGAGGTCTATGGTACTCCCACATGGGATGGATGCTTTGAAGCCA 700
||| ||| ||| ||| |||
651 CTAGAAGAGGACTTGGTACTCTCATATGGGATGGATGCTTCTTAAGCCT 700
||| ||| ||| |||
701 AATCCAAAATACAAGGCTAGAGCTGATATTACCGATATGACTGATGATTG 750
||| ||| ||| ||| |||
701 AACCCCTAAGTACAAGGCTAGAGCTGATATCACTGATATGACTGATGATTG 750
||| ||| ||| |||
751 GACCATTAGATTCCAACACAGACACTACATCTTGGTATGTTATTACCG 800
||| ||| ||| ||| |||
751 GACTATCAGATTCCAACATAGACATTACATCtTgCTcATGCTcCTTACTG 800
||| ||| ||| |||
801 CTTTCGTCAATTCCAACTCTTATCTGTGGTTACTTTCAACGACTATATG 850
||| ||| ||| |||
801 CTTTCGTgATCCCCTACTCTCATCTGTGGATACCTTCTCAACGATTACATG 850
||| ||| ||| |||

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The *pl-ole1* synthetic gene contains no intron-like regions, or predicted splice sites within its sequence. Moreover, comparing the codon usage of *Arabidopsis* with that of *Brassica napus*, *Phaseolus*

5 *vulgaris* or *Ze a mays*, with the exception of cystein (a
rare amino acid that comprises 1.7% of all *Arabidopsis*
codons, and occurs 4 times (0.8%) in *OLE1*), the sequence
contains no rare codons for any of those species. The
codon usage of *pl-ole1* is particularly similar to the
10 preferred usage of *Brassica napus*. Accordingly, *pl-ole1*
is expected to be particularly well expressed in all
those species, and well expressed in any plant species.

An alternative version of *pl-ole1*, referred to herein as *pl-ole1-2*, was also constructed. This synthetic gene was modified only in specific codons identified as high frequency splicing signals. It was discovered that this construct is expressed equally as well as *pl-ole1* in *Arabidopsis*.

25 A modification of a transformation protocol of
Pam Green (<http://www.bch.msu.edu/pamgreen/vac.html>) was
used for the transformation of *A. thaliana* with *pl-ole1*.

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The protocol was adapted from protocols by Nicole Bechtold and Andrew Bent. This protocol gives very good results, with 95% of all infiltrated plants giving rise to transformants, and a transformant in up to 1 in 25 seeds.

PROTOCOL:

1. Seeds of *Arabidopsis thaliana* ecotype Columbia were sown in lightweight plastic pots prepared 10 in the following way: mound *Arabidopsis* soil mixture into 3 to 4 inch pots, saturate soil with *Arabidopsis* fertilizer, add more soil so that it is rounded about 0.5 above the edge.

2. Plants were grown under conditions of 16 hours light / 8 hours dark at 20°C, fertilizing with *Arabidopsis* fertilizer once a week from below, adding about 0.5 L to each flat. After 4-6 weeks, plants were considered ready for vacuum infiltration when primary inflorescence was 10-15 cm tall and the secondary 20 inflorescences appeared at the rosette. The bolts were clipped back and 2 to 3 days was allowed for them to regrow before infiltration.

3. In the meantime, the construct was transformed into *Agrobacterium tumefaciens* strain 25 (LBA4404). When plants were ready to transform, a 50 mL culture of LB medium containing 50 mg/L kanamycin and 50 mg/L of streptomycin was inoculated with a 1 mL overnight starter culture.

4. Cultures were grown overnight at 28° C with 30 shaking. The culture was pelleted, the supernatant removed, and the pellet resuspended in 250 ml of infiltration medium to OD600 >0.8. Infiltration medium (1 liter) comprised 2.2 g MS salts, 1 X B5 vitamins, 50 g

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sucrose, 0.5 g MES, pH to 5.7 with KOH, 0.044 =B5M benzylaminopurine, 200 =B5L Silwet L-77 (OSI Specialties).

5. The resuspended culture was placed in a magenta jar inside a large bell jar. Pots containing plants to be infiltrated were inverted into the solution so that the entire plant was covered, including rosette, but none of the soil was submerged.

6. A vacuum of 400 mm Hg (about 17 inches) was drawn. Once the vacuum level was reached, the suction was closed and the plants allowed to remain under vacuum for five minutes. The vacuum was then quickly released. The pots were briefly drained, then placed on their sides in a tray, which was covered with a humidome to maintain humidity. The next day, the plants were removed to the growth room, the pots uncovered and set upright. Plants infiltrated with different constructs were kept separated in different trays thereafter.

7. Plants were allowed to grow under the same conditions as before. Plants were staked individually as the bolts grew. When plants were finished flowering, water was gradually reduced, then eliminated to allow the plants to dry out. Seeds were harvested from each plant individually.

25. Large selection plates were prepared: 4.3 g/L MS salts; 1 X B5 vitamins (optional); 1 % sucrose; 0.5 g/L MES pH to 5.7 with KOH; 0.8% phytagar - Autoclaved, then added antibiotics (35 μ g/mL kanamycin and 250 μ g/mL of carbenicillin) and 150 X 15 mm plates 30 were poured.

9. Plates were dried well in the sterile hood before plating - 20-30 minutes with the lids open was usually sufficient.

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10. For each plant, up to 100 μ L of seeds (approximately 2500 seeds) was sterilized and plated out individually. Seeds were sterilized as follows: 1 min in 70% ethanol, 7 minutes in 50% bleach / 0.02 % Triton 5 X-100 with vortexing, 6 rinses in sterile distilled water. Seeds were resuspended in 2 mL sterile 0.1% agarose and poured onto large selection plates as if plating phage. Plates were tilted so seeds were evenly distributed, and allowed to sit 10-15 minutes, during 10 which time the liquid soaked into the medium. Plates were sealed with Parafilm and placed in a growth room.

11. After 7 to 10 days, transformants were visible as dark green plants. These were transferred onto "hard selection" plates (100 x 15 mm plates with 15 same recipe as selection plates but with 1.5 % phytagar) to eliminate any pseudo-resistants, then replaced in the growth room.

12. After 10 to 14 days, the plants possessed at least two sets of true leaves. At this point, plants 20 were transferred to soil, covered with plastic, and moved to a growth chamber with normal conditions. They were typically kept covered for several days.

References:

25 Bechtold N, Ellis J, Pelletier G (1998) Methods Mol Biol. 82: 259-266.

Bent A, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ (1994) Science 265: 1856-1860.

30 Koncz C, Schell J (1986) Mol. Gen. Genet. 204: 383-396.

Solutions:

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1000X B5 vitamins (10 mL):

1000 mg myo-inositol

100 mg thiamine-HCl

10 mg nicotinic acid

5 10 mg pyridoxine-HCl

Dissolve in ddH₂O and store at -20 °C.

Arabidopsis fertilizer (10 liters):

10 50 mL 1M KNO₃

25 mL 1M KPO₄ (pH 5.5)

20 mL 1M MgSO₄

20 mL 1M Ca(NO₃)₂

5 mL 0.1M Fe.EDTA

10 mL micronutrients (see below)

15 Dissolve in ddH₂O and store at room temperature

Arabidopsis micronutrients (500 mL):

20 70 mL 0.5M boric acid

14 mL 0.5M MnCl₂

2.5 mL 1M CuSO₄

1 mL 0.5M ZnSO₄

1 mL 0.1M NaMoO₄

1 mL 5M NaCl

0.05 mL 0.1M CuCl₂

25 Dissolve in ddH₂O and store at room temperature

EXAMPLE 3

Customizing OLE1 to Express Post-Translational Modifications

30

After determining the optimized codon preferences of OLE1 mRNA (or mRNA derived from another fungal or animal desaturase) for high level expression in the host plant, specific amino acids that are involved in

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the post-translational control of enzyme activity or stability are altered to maximize the catalytic activity of the expressed enzyme. There are a number of protein kinase and/or phosphorylase consensus sequences that are 5 highly conserved in the fungal and animal desaturases. These are shown below. First is shown a table of aligned potential phosphorylation sites in desaturases. Next is shown a pileup of Δ -9 fatty acid desaturases. PROSITE analysis of these desaturases predicts a number of 10 potential phosphorylation sites, highlighted by bold underlined characters.

Table 2 - Aligned Potential phosphorylation sites in desaturases. Phosphorylation sites are indicated with respect to amino acid positions in the Oleip protein coding sequence.
 Abbreviations, S = serine, T = Threonine, A=Alanine, PKC - protein kinase C like; CK-2 - casein kinase II like; CAMP - cAMP activated kinase (PKA) like; np., not predicted as a phosphorylation site.

Residue position	Oleip Residue	Oleip sequence	Phosphorylation type	Fungal/b5	Animal	Insect
166	S	SHR	PKC	mixed, S or A	all S, SHR	N.P., A
169	T	np	PKC	S or A	all TYK or SYK	
191	S	SAK	PKC	all S	all D	all A
206	T	np	CK-2	all T	all S	all S
208	T	TLRD	CK-2	all T	all T	all T
215	A	np	PKC	A or V	all S	all A
323	T	TPRD	CK-2	T or S	S or np	np
351	R	np	CK-2	all R	all S	all K
383	S	KKFS	CAMP	S or np	all S	S but np

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Pileup of Δ -9 fatty acid desaturases showing potential phosphorylation sites:

	1	50
Rat	-----	-----MPAHM LQE.ISSSY.
Mouse	-----	-----MPAHM LQE.ISSSY.
Sheep	-----	-----MPAHL LQEEISSSY.
Pig	-----	-----SSY.
Human	-----	-----MPAHL LQDDISSSY.
Hamster	-----	-----MPGHL LQEEMTSSYT
Drosophila	-----	-----MPP NAQAGAQSI
Moth	-----	-----
C.elegans	-----	-----MTVKTRSN LAKKIEKDGG
S.cerevisiae	MPTSGTTIEL	IDDQFPKDDS ASSGIVDEVD LTEANILATG LNKKAPRIVN
P. angusta	-----	-----
H. capsulatum	-----	-----
M. rouxii	-----	-----
C. curvatus	-----	-----
C. merolae	-----	-----MTAKVESKVR EEEKGSNPST
	51	100
Rat	TTTTTITEPP	SGNLQNGREK MKKPLYLEE DI.....RPE MREDIHDPSY
Mouse	TTTTTITAPP	SG...NEREK VKTVPLHLEE DI.....RPE MKEDIHDPTY
Sheep	TTTTTITAPP	SRVLQNGGGK LEKTPLYLEE DI.....RPE MRDDIYDPNY
Pig	TTTTTITAPS	SRVLQNGGGK SEKTPQYVEE DI.....RPE MKDDIYDPTY
Human	TTTTTITAPP	PGVLQNGGDK LETMPLYLED DI.....RPE IKDDIYDPTY
Hamster	TTTTTITEPP	SESLQ..... KTVPLYLEE DI.....RPE MKEDIYDPSY
Drosophila	DSLIAASAA	ADAGQSPTKL QEDSTGVLFE CD.....VET TDGLLVKDIT
Moth	-----	-----MPPQG QTGGSWVLYE TD.....AVN TDTD..APVI
C.elegans	PETQYLAQDP	NEIIQLQEES KKVPKCLPA RLPTAACKAS QENGECQKIV
S.cerevisiae	GFGSLMGSKE	MVSVEFDKKG NEKKSNLDRD LEKDNQEKEE AKTKIH.ISE
P. angusta	-----MGTKS	MTDVTAAEL. .SKDSVAMM LAKDRELKNK YLKQKH.ISE
H. capsulatum	-----MA	LNEAPTASPV AETAAGGKDV VTDAARRPNS EPKKVH.ITD
M. rouxii	-----	-----MSN IATLTSTART KTESMKPPLP KTKMPP.LFD
C. curvatus	~MSASTKQAS	TTVAQPSGKP VTNVIDPERD DFIVPDNYVT RTVENM.KML
C. merolae	AAADDGAVI	PTLKPRPKPA VEPLEREGVE FDPQRGLVFE KTRSSKWMSE
	101	150
Rat	QDEEGPPPKL	EYVWRNIILM ALLHVGALYG ITL.IPSSKV YTLLWGIFY
Mouse	QDEEGPPPKL	EYVWRNIILM VLLHGGLYG IIL.VPSCKL YTALFGIFY
Sheep	QDKEGPKPKL	EYVWRNIILM GLLHGLALYG ITL.IPTCKI YTFLWVLFYY
Pig	QDKEGPQGKL	EYVWRNIILM SLLHGLALYG IIL.IPTCKI YTLLWAFAYY
Human	KDKEGPSPKV	EYVWRNIILM SLLHGLALYG ITL.IPTCKF YTWLWGVFYY
Hamster	QDEEGPPPKL	EYVWRNIILM ALLHGLALYG LVL.VPSSKV YTLLWAFVYY
Drosophila	VMKKAEKRL	KLVWRNIIAF GYLHLAALYG AYLMVTSAKW QTCILAYFLY
Moth	VPPSAEKREW	KIVWRNVILM GMLHIGGVYV AYLFLTKAMW LTDLFaffly
C.elegans	FLEIVIPYKM	EIVWRNVALF AALHFAAAIG LYQLIFEAKW QTVIFTFLY
S.cerevisiae	QPWTLNWHR	QPWTLNWHR HLNWLNMVLV CGMPMIGWYF ALSGKVPLHL NVFLFSVFYY
P. angusta	QPWTWENWHR	QPWTWENWHR HINWLNFILV LAVPFAG..L ISTKWVPLKL HTFVTAVILY
H. capsulatum	TPITLANWHR	TPITLANWHR HISWLNTLI IAIPIYG..L VQAYWVPLHL KTALWAVVYY
M. rouxii	QPVTSKNWT	QPVTSKNWT FVNWPQAILL CTVPLIALYG IFT..TELTK KTLIWSWIYY
C. curvatus	PPVTWRNLHK	PPVTWRNLHK NIQWISFLAL TIPPAMAIYG LCT..VPVQT KTFIWSVYY
C. merolae	KELNELPLLQ	KELNELPLLQ RINWLS.TSI IFTPLIGT.L IGIWFVPLQR KTLVLAIVTY

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	151	200
Rat	LISALGITAG AHRLWSHRTY KARLPLRIFL IIANTMAFQN DVYEWARDHR	
Mouse	MTSALGITAG AHRLWSHRTY KARLPLRIFL IIANTMAFQN DVYEWARDHR	
Sheep	VISALGITAG VHRLWSHRTY KARLPLRVFL IIANTMAFQN DVFEWSRDHR	
Pig	LLSAVGVTAG AHRLWSHRTY KARLPLRVFL IIANTMAFQN DVYEWARDHR	
Human	FVSALGITAG AHRLWSHRSY KARLPLRLFL IIANTMAFQN DVYEWARDHR	
Hamster	VISIEGIGAG VHRLWSHRTY KARLPLRIFL IIANTMAFQN DVYEWARDHR	
Drosophila	VISGLGITAG AHRLWAHRSY KAKWPLRVL VIFNTIAFQD AAYHWARDHR	
Moth	LCSGLGITAG AHRLWAHKSY KARLPLRLLL TLFNTLAFQD AVIDWARDHR	
C. elegans	VFCGGFGITAG AHRLWSHKSY KATTPMRIFL MILNNIALQN DVIEWARDHR	
S.cerevisiae	AVGGVSITAG YHRLWSHRSY SAHWPLRLFY AIFGCASVEG SAKWWGHSHR	
P. angusta	CFGGISITAG YHRHWAHRAY DCKLPVKIFF ALFGASAVEG SIKMWGHQHR	
H. capsulatum	FMTGLGITAG YHRLWAHCSY SATLPLKIYL AAVGGGAEVG SIRWWARGHR	
M. rouxii	FITGLGITAG YHRMWSHRAY RGTDLLRWFM SFAGAGADEVG SIYWWSRGHR	
C. curvatus	FITGLGITAG YHRLWAHRSY NASKPLQYFL ALCGAGSVQG SIRWWSRGHR	
C. merolae	FCCGLGITCG YHRLWSHRSY EAHWLVQVIL ACFGAAAFEG SARYWCRLHR	
	201	250
Rat	AHHKFSETHA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGG KLDMSDLKAE	
Mouse	AHHKFSETHA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGG KLDMSDLKAE	
Sheep	AHHKFSETDA DPHNSRRGFF FSHVGWLLVR KHPAVREKGA TLDLSDLRAE	
Pig	AHHKFSETDA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGG LLNMSDLKAE	
Human	AHHKFSETHA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGS TLDLSDLLEAE	
Hamster	AHHKFSETYA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGG KLDMSDLKAE	
Drosophila	VHHKYSETDA DPHNATRGFF FSHVGWLLCK KHPEVKAKGK GVDLSDLRAD	
Moth	MHHKYSETDA DPHNATRGFF FSHVGWLLVR KHPQIKAKGH TIDLSDLKSD	
C. elegans	CHHKWTDTDA DPHNTTRGFF FAHMGWLLVR KHPQVKEQGA KLDMSDLLSD	
S.cerevisiae	IHHRYTDTLR DPYDARRGLW YSHMGWMLLK PNP...KYKA RADITDMTDD	
P. angusta	VHHRYTDTPR DPYDAKRGFW YSHMGWMLLV PNP...RYKA RADISDLDD	
H. capsulatum	AHHRYTDTDK DPYSVRKGLL YSHIGWMVMK QNP...KRIG RTEITDLNEQ	
M. rouxii	AHHRWTDTDK DPYSAHRGFF FSHFGWMLVQ RPK...NRIG YADVADLKAD	
C. curvatus	AHHRYTDTKL DPYSAHEGFH HAHMGWMLI. KPR...GKIG VADISDLSKN	
C. merolae	AHHRYVDSDR DPYAVEKGFW YAHLWWMVFK LPR...QRQG RVDIRDLNAN	
	251	300
Rat	KLVMFQRRYY KPGLLL MCFI LPTLVPWYCW GETFLHSLFV STFLRYTLVL	
Mouse	KLVMFQRRYY KPGLLL MCFI LPTLVPWYCW GETFVNNSLFV STFLRYTLVL	
Sheep	KLVMFQRRYY KPGVLLLCFI LPTLVPWYFLW GESFQNSLFF ATFLRYAVVL	
Pig	KLVMFQRRYY KPGILL MCFI LPTIVPWYCW GEAFPQSLFV ATFLRYAIVL	
Human	KLVMFQRRYY KPGLLMMCFI LPTLVPWYFW GETFQNSVFV ATFLRYAVVL	
Hamster	KLVMFQRRYY KPAILL MCFI LPTFVPWYFW GEAFVNSLCV STFLRYTLVL	
Drosophila	PILMFQKKYY MILMPIACFI IPTVVPYMW GESFMNAWFV ATMFRWCFL	
Moth	PILRFQKKYY LTLMPLICFI LPSYIPT.LW GESAFNAFFV CSIFRYVYVL	
C. elegans	PVLVFQRKHY FPLVILCCFI LPTIIPVYFW KETAIFIY AGTFRYCFTL	
S.cerevisiae	WTIRFQHRHY ILLMLLTAFV IPTLICGYFF ND.YMGGGLIY AGFIRVFVIQ	
P. angusta	WVVRVQHRHY LLLMVVMAFL FPAVLTHYLW ND.FWGGFIY AGLLRAVVIQ	
H. capsulatum	PVVVWQHRNY LKVVIFMGIV FPMLVSGLGW GD.WFGGFIY AGILRIFVVQ	
M. rouxii	HVVAFQHKYY PYFALGMGFI FPTLVAGLGW GD.FRGGYFY AGVRLLCFVH	
C. curvatus	PVVVKWQHNNY VALLFMGLA FPTLVAGLGW GD.WWGGLFF AGAARLTVFVH	
C. merolae	PILRFQHRYY LQIAILFSFV IPTLTISSLW GD.FWGGLVY ACLGRMLFVQ	

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	301	350
Rat	NATWLVNSAA HLYGYR PYDK NIQSRENILV SLGSVGE GFH NYHHAFPYDY	
Mouse	NATWLVNSAA HLYGYR PYDK NIQSRENILV SLGAVGE GFH NYHHTFPFDY	
Sheep	NATWLVNSAA HMYGYR PYDK TINPRENILV SLGAVGE GFH NYHHTFPDY	
Pig	NATWLVNSAA HLYGYR PYDK TISPRENILV SLGAVGE GFH NYHHTFPDY	
Human	NATWLVNSAA HLFGYR PYDK NISPRENILV SLGAVGE GFH NYHHSFPDY	
Hamster	NATWLVNSAA HLYGYR PYDK NIDPRENALV SLGCLGE GFH NYHHAFPYDY	
Drosophila	NVTWLVNSAA HKFGGR PYDK FINPSENISV AILAFGE GWH NYHHVFPWDY	
Moth	NVTWLVNSAA HLWGSKPYDK NINPVETRPV SLVVLGE GFH NYHHTFPWDY	
C. elegans	HATWCINSAA HYFGWK PYDS SITPVENVFT TIAAVGE GGH NFHHTFPQDY	
S.cerevisiae	QATFCINSLA HYIGTQPFDD RRTPRDNWIT AIVTFGE GYH NFHHEFPPTDY	
P. angusta	QATFCVNSLA HWIGEQPFDD RRTPRDHVLT ALVTFGE GYH NFHHEFP PSDY	
H. capsulatum	QATFCVNSLA HWLGDPQPFDD RNSPRDHIVT ALVTLGE GYH NFHHEFP PSDY	
M. rouxii	HATFCVNSLA HYLGE STFDD HNTPRDSWVT ALVTMGE GYH NFHHQFPQDY	
C. curvatus	HSTFCVNSLA HWLGETPF DN KHTPKDHFIT ALVTVGE GYH NFHHQFPMDF	
C. merolae	QSTFCVNSLA HWWGEQTF SR RHTSYDSVIT ALVTLGE GYH NFHHEFPHDY	

	351	400
Rat	SASEY.RWHI NFTTFFIDCM AALGLAYDRK KVSKAAVLAR IKRTGDGSHK	
Mouse	SASEY.RWHI NFTTFFIDCM AALGLAYDRK KVSKATVLAR IKRTGDGSHK	
Sheep	SASEY.RWHI NFTTFFIDCM AAIGLAYDRK KVSKAAVLGR MKRTGEESYK	
Pig	SASEY.RWHI NLTTFFIDCM AALGLAYDRK KVSKAAIL-- -----	
Human	SASEY.RWHI NFNTFFIDWM AALGLTYDRK KVSKAAILAR IKRTGDGNYK	
Hamster	SASEY.RWHI NFTTFFIDCM AALGLAYDRK KVSKAAVLAR IKRTGDGSCK	
Drosophila	KTAEFGKYSL NFTTAFIDFF AKIGWAYDLK TVSTDIIKKR VKRTGDGTHA	
Moth	KTAELGDYSL NFTKMFIDFM ASIGWAYDLK TVSTDVIQKR VKRTGDGSHA	
C. elegans	RTSEYS.LKY NWTRVLIDTA AALGLVYDRK TACDEIIGRQ VSNHGCDIQR	
S.cerevisiae	RNA.IKWYQY DPTKVIIYLT SLVGLAYDLK KFSQNAIEEA LIQQEQKKIN	
P. angusta	RNA.LKWYQY DPTKVVIYLL SKVGLAYNLK KFSQNAIDQG ILQQQQKKLD	
H. capsulatum	RNA.IEWHQY DPTKWITIWIW KQLGLAYDLK QFRANIEIEKG RVQQLQKKID	
M. rouxii	RNA.IKFGQY DPTKWKIIIVL SWFGLAYELK QFPTNEVTKG RLFMEEKRIQ	
C. curvatus	RNA.IKWYQY DPTKWFIVTM AQLGLASHLK KFPDNEIKKG QYTMKLMQLQ	
C. merolae	RNG.VVWYHW DPTKWWIRLL SWAGLAWHLV RFPRNELVKA RLQVRQEILD	

	401	450
Rat	SS*----- ----- ----- ----- ----- ----- -----	
Mouse	SS----- ----- ----- ----- ----- ----- -----	
Sheep	SG----- ----- ----- ----- ----- ----- -----	
Pig	----- ----- ----- ----- ----- ----- -----	
Human	SG----- ----- ----- ----- ----- ----- -----	
Hamster	SG----- ----- ----- ----- ----- ----- -----	
Drosophila	TWGWDVDQ PKEEIE.DAVI THKKSE----- ----- -----	
Moth	VWGDDHEVH QEDKKLAAII NPEKTE----- ----- -----	
C. elegans	GKSIM----- ----- ----- ----- ----- ----- -----	
S.cerevisiae	KKKAKINWGP VLTDLPMWDK QTFLAKS.KE NKGLVIISGI VHDVSGYISE	
P. angusta	RMRRAKLNWGP QLSELPVWDK STFFEKA.KE QKGLVIISGI VHDCANFLTE	
H. capsulatum	QRRAKLDWGI PLEQLPVVIEW DDYVDQA.KN GRGLIAIAGV VHDVTDFIKD	
M. rouxii	AQKAKLISYGT PLKDLPIYT WEEYQSLVLND NKKWVILIEGV LYDVEEFMKE	
C. curvatus	EQSEKLEWPK HSNDLPPVISW EDFQA..ESK TRALIAVHGF IHDCSSFIED	
C. merolae	EAKKRVWDWK PIESLPVWTW KDVQRSLAKEE NRLLVVIEGI VHDCTRFKVQ	

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	451	500
Rat	-----	-----
Mouse	-----	-----
Sheep	-----	-----
Pig	-----	-----
Human	-----	-----
Hamster	-----	-----
Drosophila	-----	-----
Moth	-----	-----
C. elegans	-----	-----
S. cerevisiae	HPGGETLIKT ALGKDATKAF SGGVYRHSNA AQNVLADMVR AVIKESKN	SA
P. angusta	HPGGQALLKT SFGKDATMAF NGGVYAHNSNA AHNLLATMRV AVIRDGGANG	
H. capsulatum	HPGGKAMINS GIGKDATAMF NGGVVNHSNA AHNQLSTMRV G VIRGGCEVE	
M. rouxii	HPGGMKYLST AVGKDMTTAF NGGIYVHSNG TRNLLTSLRV G VLRNGMQV.	
C. curvatus	HPGGAHLIK R AIGTDSTTAF FGGVYDHSNA AHNLLAMMRV G VLDGGMEVE	
C. merolae	HPGGQRILEF WNVRDATQAF NGDVYNHTKA ARNLLAHLRV AQLKEIYEPE	

Protein kinase (specifically cAMP- and cGMP-dependent) phosphorylation sites. There have been a number of studies relative to the specificity of cAMP- and cGMP-dependent protein kinases (Fremisco J.R. et al., J. 5 Biol. Chem. 255:4240-4245, 1980; Glass D.B., Smith S.B., J. Biol. Chem. 258:14797-14803, 1983; Glass D.B. et al., J. Biol. Chem. 261:2987-2993, 1986). Both types of kinases appear to share a preference for the phosphorylation of 10 serine or threonine residues found close to at least two consecutive N-terminal basic residues. It is important to note that there are quite a number of exceptions to this rule. However, the consensus pattern is as follows: 15 [RK] (2)-x-[ST], where S or T is the phosphorylation site.

Protein kinase C phosphorylation site. *In vivo*, 20 protein kinase C exhibits a preference for the phosphorylation of serine or threonine residues found close to a C-terminal basic residue (Woodget J.R. et al., Eur. J. Biochem. 161:177-184, 1986; Kishimoto A. et al., J. Biol. Chem. 260:12492-12499, 1985). The presence of additional basic residues at the N- or C-terminus of the target amino acid enhances the V_{max} and K_m of the

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phosphorylation reaction. The consensus pattern is:
[ST]-x-[RK] where S or T is the phosphorylation site.

Casein kinase II phosphorylation site. Casein kinase II (CK-2) is a protein serine/threonine kinase whose activity is independent of cyclic nucleotides and calcium. CK-2 phosphorylates many different proteins. The substrate specificity (Pinna L.A., Biochim. Biophys. Acta 1054:267-284, 1990) of this enzyme can be summarized as follows: (1) Under comparable conditions Ser is favored over Thr; (2) an acidic residue (either Asp or Glu) must be present three residues from the C-terminal end of the phosphate acceptor site; (3) additional acidic residues in positions +1, +2, +4, and +5 increase the phosphorylation rate (most physiological substrates have at least one acidic residue in these positions); (4) Asp is preferred to Glu as the provider of acidic determinants; and (5) a basic residue at the N-terminus of the acceptor site decreases the phosphorylation rate, while an acidic one will increase it. The consensus pattern is: [ST]-x(2)-[DE] where S or T is the phosphorylation site (note: this pattern is found in most of the known physiological substrates).

If phosphorylation of a specific site by any kinase is found to increase the catalytic activity or stability of the encoded desaturase protein, the phosphorylated serine or threonine residue is changed to encode a negatively charged amino acid (aspartic acid or glutamic acid) in order to permanently optimize the activity or the protein. If phosphorylation of a specific residue is found to decrease the activity or stability of the encoded desaturase, the affected serine or threonine encoding codon is altered to substitute a neutral or a positively charged amino acid that will permanently optimize the activity or stability of the protein.

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EXAMPLE 4

Further Modifications and Improvements of the
Saccharomyces cerevisiae OLE1 Gene for Plant Expression
5 Using Elements Derived from Native Plant Desaturase Genes

The activity of the native or modified forms of the *Saccharomyces cerevisiae OLE1* Δ -9 desaturase gene in plant tissues may be further improved by the substitution 10 or inclusion of elements derived from native plant desaturase genes. Favorable plant gene elements may include sequences that improve the expression of the modified gene at one or more levels, including the following: 1) transcription, 2) pre-mRNA processing, 3) 15 mRNA transport from the nucleus to the cytoplasm, 4) mRNA stability 5) translation, 6) targeting or retention of the protein at the appropriate membrane surface or organelle surface, 7) protein folding and maturation, and 8) stability of the functional desaturase protein.

20 The inventors have shown that the *OLE1* gene can tolerate significant modifications without losing its biological activity. These modifications include deletion of the "coiled coil" region, the addition of 239 amino acids to the N-terminus of *OLE1p* and truncation of 55 and 25 60 amino acids from the N-terminal end of the protein. The inventors have also shown that modifications of the 5' and 3' untranslated regions of the *OLE1* mRNA can significantly affect its stability. For example, removing a short open reading frame near the 5' "cap" region of the *OLE1* mRNA 30 increases its half-life in *Saccharomyces* from 12 minutes to approximately 25 minutes. The existence of elements in the mRNA that affect its stability indicate that other elements might also exist that affect the stability of an mRNA generated by a synthetic gene in another host organism.

35 Plant desaturase gene elements that enhance the

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function of the modified Δ -9 desaturase gene are identified by a 2-step method. STEP 1 involves isolating a series of DNA sequences from a cDNA that encodes a plant ER lipid biosynthetic enzyme. Those elements are linked, or 5 inserted into regions of a native or "optimized" gene under control of a yeast promoter in a vector suitable for expression in *Saccharomyces cerevisiae*. The resulting vectors are then tested for their ability to produce functional desaturase enzymes in strains of *Saccharomyces* 10 that contain an inactive form of the Δ -9 fatty acid desaturase gene.

In STEP 2, plant desaturase sequences from the above vectors that are found to produce a functional Δ -9 desaturase gene are used to isolate homologous sequences 15 from plant genomic DNA. The isolated genomic sequences are used to construct a synthetic gene that produces an mRNA that encodes the same functional desaturase protein produced by the vector in step 1. In this instance, the genomic sequences encompass the same protein coding 20 elements as those encoded by the homologous cDNA sequence and also include genomic elements that encode the 5' and/or 3' untranslated regions of the plant desaturase mRNA. These combined genomic elements should differ from the cDNA derived sequences used in STEP 1 by containing authentic 25 plant introns, (which may facilitate efficient and correct splicing of the chimeric mRNA in the plant nucleus) and signals that affect the mRNA stability, mRNA transport, and efficient translation of the mRNA in plant tissues. The chimeric plant / synthetic gene containing the genomic 30 sequences is inserted into vectors under the control of plant seed-specific promoters and tested for expression and desaturase function in plants, including *Brassica*, *Arabidopsis*, maize and soybeans.

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The following specific examples further illustrate these methods employing the *Arabidopsis FAD2* gene, which encodes an ER Δ 12-desaturase, as a source of plant desaturase DNA sequences. In the preferred 5 embodiment, the source of the plant desaturase DNA would be the *FAD2* homolog, or a related ER lipid biosynthetic gene, that is derived from the same plant species that is intended to be modified by the resulting vector for commercial use.

10

A. Substitution of the N-terminal *OLE1* protein coding sequences and with N-terminal sequences from the derived from the *Arabidopsis FAD2* gene.

15 1) A cDNA containing the *FAD2*, Δ -12 desaturase, mRNA coding sequence is isolated by reverse transcriptase - polymerase chain reaction (RT-PCR) of isolated mRNAs derived from *Arabidopsis* tissue or by direct DNA synthesis using the protein and DNA sequences set forth in SEQ ID NO:4 and SEQ ID NO:5 (open reading frame starts at +93).
20 2) The inventors have shown that substitution of transmembrane sequences of the *OLE1* gene with transmembrane sequences from the *Saccharomyces FAH2* gene abolishes Δ -9 desaturase activity. *FAH2* encodes a sphingolipid fatty acid hydroxylase, which is an ER membrane protein.
25 TMPredict analysis of the *Arabidopsis FAD2* sequence indicates that the first transmembrane region of its encoded protein begins at residue +52 and a similar analysis of the *OLE1* sequence indicates that its first transmembrane sequence begins at residue +113. Because the 30 inclusion of potential membrane spanning elements from the plant desaturase could produce significant changes in the desaturase core enzyme structure that affect activity, only sequences encoding residues +1 to +52 of *FAD2* are tested

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for functional linkages or substitutions in the 113 residue N-terminal region of *OLE1*.

A series of PCR oligonucleotide primers are synthesized that include a 5' primer that complements sequences including +1 start codon of the *FAD2* gene and 3' primers that complement sequences ending, for example, at residues +20, +35 and + 52 of the *FAD2* gene. These are used to amplify a series of fragments of different lengths from the *FAD2* cDNA that extend from the +1 codon through codon +52. A second PCR amplification is performed using a 5' primer that is complementary to sequences that include the 5' end of the *FAD2* mRNA and the 3' primer that includes codon +52. That amplification is done using *Arabidopsis* genomic DNA as a template. The amplified fragment from that reaction is cloned into a bacterial vector and subjected to DNA sequencing to detect the presence of introns within the genomic sequence. The cloned genomic fragment is also used to construct vectors for plant expression as indicated in STEP 2 of the method.

The amplified cDNA fragments is inserted into yeast expression vectors that contain the native *OLE1* mRNA coding sequence under the control of the *Saccharomyces* galactose inducible, *GAL1* promoter. Insertion of the plant DNA fragments can be done in several ways: 1) A fragment is inserted upstream of the *OLE1* protein coding sequences so that its protein coding element is fused in frame to the +1 codon of the *OLE1* encoded protein, 2) the codons on the plant fragment could replace the equivalent *OLE1* residues starting from the +1 ATG codon (e.g. a plant DNA fragment containing codons +1 -> +52 replaces *OLE1* codons +1 -> +52) and 3) the full length fragment containing codons +1 -> + 52 of the plant gene is fused in frame to codon +114 of the *OLE1* gene, replacing the *OLE1* residues +1 -> +113 with

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plant desaturase residues +1 -> +52.

The resulting plasmids are transformed into a haploid *ole1Δ::LEU2* strain of *Saccharomyces*. That strain contains a null, disrupted form of the *OLE1* gene and therefore has a growth requirement for unsaturated fatty acids. The transformed *Saccharomyces* strains are grown on fatty acid depleted galactose medium to test for the ability of the induced chimeric gene to support growth of the strain without fatty acids. Transformed strains that grow on the fatty acid deficient medium are further analyzed to assess the effects of the plant sequences on desaturase function. This is done by Western blot analysis, to measure levels of the resulting desaturase protein and by fatty acid analysis of total cellular lipids, to assess the relative activity of the desaturase enzyme by comparison of the ratio of saturated to unsaturated fatty acids.

3) Using information derived from the above tests, a chimeric desaturase gene is constructed using the amplified genomic DNA from the *FAD2* gene. Construction, testing, and analysis these vectors is guided by the principle that the most desirable vector is one that maximizes the use of the plant gene sequences and minimizes the use of the *Saccharomyces* Δ-9 desaturase gene sequences while retaining optimal desaturase function. Plant DNA fragments derived from the genomic DNA amplification that extend from the 5' end of the mRNA sequence to the longest sequence that produces optimal desaturase function in yeast are inserted into a vector containing the native Δ-9 desaturase gene (or one of its modified forms produced by the methods described above). The fragment is inserted into the vector so that the 3' end of its protein coding sequence produces an mRNA that generates a protein sequence

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identical to its counterpart derived from the *FAD2* cDNA sequences. The resulting chimeric desaturase gene, which now encodes an mRNA that includes the *FAD2* 5' untranslated region in addition to the modified protein coding sequences, is placed into a plant expression vector under the control of a suitable plant promoter and plant termination/ polyadenylation sequences.

4) The resulting vectors containing the plant/yeast chimeric desaturase sequences are transformed into plants for testing and analysis of desaturase function. Suitable test plants include *Arabidopsis thaliana*, and *Brassica napis*. A method for transformation and analysis of desaturase gene expression in *Arabidopsis* is provided above. A method for transformation and analysis of yeast desaturase expression in *Brassica napis* is described in U.S. Patent No. 5,777,201 to Poutre et al. (incorporated by reference herein).

B. Insertion or substitution of *Arabidopsis FAD2* C-terminal protein coding sequences and 3' mRNA untranslated region sequences into native and modified forms of the *OLE1* gene.

The inventors have previously shown that proteins encoded by the *Saccharomyces ELO2* and *ELO3* genes contain a series of charged residues in their C-terminal region. These proteins are located on the ER surface and function in the biosynthesis of very long chain fatty acids as described in Oh et al. (J. Biol. Chem. 272: 17376-17384, 1997) (incorporated by reference herein). They further showed that deletion of the region containing the charged residues causes the proteins to be mislocalized from their normal cellular locations in the endoplasmic reticulum, resulting in reduced function. Similar clusters

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of charged residues occurs in the C-terminal region of the *OLE1* gene that are apparently associated with ER retention or localization. These residues do not appear to be a part of the functional cytochrome b_5 domain. A detailed comparison of the C-terminal *OLE1* and the *Arabidopsis FAD2* sequences show that the plant desaturase has similar, but not identical, clusters of charged residues to those in the *OLE1* gene. These sequences are shown below:

10 SEQ ID Nos: 6 and 7:

Comparison of the charged carboxyl terminal amino acids of Olelp (SEQ ID NO:7) and the Arabidopsis Fad2p desaturase (SEQ ID NO:6) (The region of the OLE1 gene shown does not appear to be a functional part of its cytochrome b₅ domain).

20 *S.cerevisiae* OLE1 MRVAVIKESK NSAIRMASKR GEIYETGKFF *

Methods similar to those shown in Section A can be used to identify *Arabidopsis* FAD2 sequences that can replace the *OLE1* C-terminal sequences to optimize gene expression, membrane targeting and ER retention of the chimeric enzyme.

1) A series of oligonucleotide primers for PCR amplification are synthesized for isolation of elements in the C-terminal region of the *FAD2* gene. A *FAD2* DNA fragment encompassing that region is generated by PCR amplification of the cDNA clone. Alternatively, given the smaller size of the fragment it or modified forms of the plant fragment may be generated directly by DNA synthesis.

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A fragment containing that region and its flanking 3' untranslated region also is generated by PCR amplification of *Arabidopsis* genomic DNA as described above. That fragment is cloned into an appropriate vector and sequenced. 5 as also described.

2) Vectors are constructed that contain the plant DNA fragments linked to or substituted into the *OLE1* C-terminal coding region as described in Section A. In this instance, the plant DNA fragments are linked in frame to 10 the carboxyl terminal residues of the *OLE1* protein coding region.

3) The resulting vectors are transformed into the *Saccharomyces ole1Δ* strain and tested for desaturase function as described in Section A.

15 4) Using information derived from the above tests, chimeric desaturase genes containing the C-terminal plant sequences that produce functional desaturases are constructed using the amplified genomic DNA from the *FAD2* gene, according to the principles outlined in Section A. 20 The resulting sequences are employed to construct vectors that will express the chimeric plant/yeast gene under control of plant promoter and plant termination/polyadenylation sequences. Those vectors are transformed 25 into plants for testing and analysis of desaturase function as described above.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the 30 scope of the appended claims.

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We claim:

1. A synthetic fatty acid desaturase gene for
5 expression in a multicellular plant, the gene comprising a
desaturase domain and a cyt b₅ domain, wherein the gene is
customized for expression in a plant cytoplasm.

2. The synthetic gene of claim 1, customized
10 from a naturally occurring gene encoding a cytosolic Δ-9
desaturase.

3. The synthetic gene of claim 2, customized
from a naturally occurring gene from *Saccharomyces*
15 *cerevisiae*.

4. The synthetic gene of claim 3, customized
from a naturally occurring gene from *Saccharomyces*
cerevisiae that encodes SEQ ID NO:2.

20 5. The synthetic gene of claim 4, customized
from a naturally occurring gene from *Saccharomyces*
cerevisiae comprising SEQ ID NO:1.

25 6. The synthetic gene of claim 3, comprising SEQ
ID NO:3.

7. The synthetic gene of claim 1, which further
comprises an expression regulatory sequence from a plant
30 gene encoding an ER biosynthetic pathway enzyme.

8. The synthetic gene of claim 1, customized for
expression in a monocotyledonous plant.

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9. The synthetic gene of claim 1, customized for expression in a dicotyledonous plant.

10. The synthetic gene of claim 1, customized
5 for expression in a plant genus selected from the group
consisting of *Arabidopsis*, *Brassica*, *Phaeoleolus*, *Oryza*,
Olea, *Elaeis* (Oil Palm) and *Zea*.

11. The synthetic gene of claim 1, customized
10 from a naturally occurring gene comprising both a
desaturase domain and a cyt b₅ domain.

12. The synthetic gene of claim 1, wherein the
gene is a chimeric gene comprising a desaturase domain and
15 a heterologous cyt b₅ domain.

13. The synthetic gene of claim 1, customized
from a naturally occurring gene such that the synthetic
gene and the naturally occurring gene encode an identical
20 amino acid sequence.

14. The synthetic gene of claim 13, wherein the
synthetic gene and the naturally occurring gene encode SEQ
ID NO:2.

25

15. The synthetic gene of claim 1, customized
from a naturally occurring gene such that the synthetic
gene and the naturally occurring gene encode a similar
amino acid sequence.

30

16. The synthetic gene of claim 1, customized
from a naturally occurring gene such that the synthetic
gene and the naturally occurring gene encode a similar

amino acid sequence, and the synthetic gene possesses improved stability or catalytic activity as compared with the naturally occurring gene.

5 17. A method for constructing a customized bifunctional desaturase/cyt b₅ encoding gene for expression in the cytosol of a multicellular plant, comprising the steps of:

- 10 (a) providing a DNA molecule comprising a desaturase-encoding moiety operably linked to a cyt b₅-encoding moiety, said DNA molecule producing the bifunctional polypeptide in a non-customized form;
- 15 (b) back-translating the polypeptide sequence using preferred codons for expression in a multicellular plant, thereby producing a back-translated nucleotide sequence;
- 20 (c) analyzing the back-translated nucleotide sequence for features that could diminish or prevent expression in the plant cytoplasm;
- 25 (d) modifying the analyzed sequence to correct or remove the features that could diminish or prevent expression in the plant cytoplasm; and
- (e) optionally, introducing pre-determined cloning features into the sequence in a manner that does not materially affect the codon usage or final polypeptide sequence, thereby producing the customized bifunctional desaturase/cyt b₅ encoding gene for expression in the cytosol of a multicellular plant.

30 18. The method of claim 17, wherein the features that could diminish or prevent expression in the plant cytoplasm include one or more features selected from the group consisting of: putative intron splice sites, plant

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polyadenylation signals, RNA polymerase II termination sequences, and hairpin consensus sequences.

19. The method of claim 17, which further
5 comprises the step of:

(f) testing the customized bifunctional desaturase/cyt b₅ encoding gene for desaturase function in fatty acid deficient strains of a microorganism prior introducing the gene into vectors for expression in plants.

10

20. The method of claim 19, wherein the microorganism is *Saccharomyces cerevisiae*.

21. The method of claim 17, which further
15 comprises incorporating into the customized gene one or more genomic segments from plant desaturase or other ER lipid biosynthetic genes, which comprise beneficial elements to further optimize expression of the genes in plants, comprising the steps of:

20 a) selecting a cDNA sequence that potentially comprises one or more of the beneficial elements;

25 b) creating a yeast vector expressing a desaturase gene modified to contain one or more of the beneficial elements;

c) testing the vector in a yeast expression system;

d) isolating regions from genomic DNA that are homologous to the beneficial elements from the cDNA;

30 and

e) operably linking the genomic DNA regions to the customized bifunctional desaturase/cyt b₅ encoding gene to produce the further customized gene.

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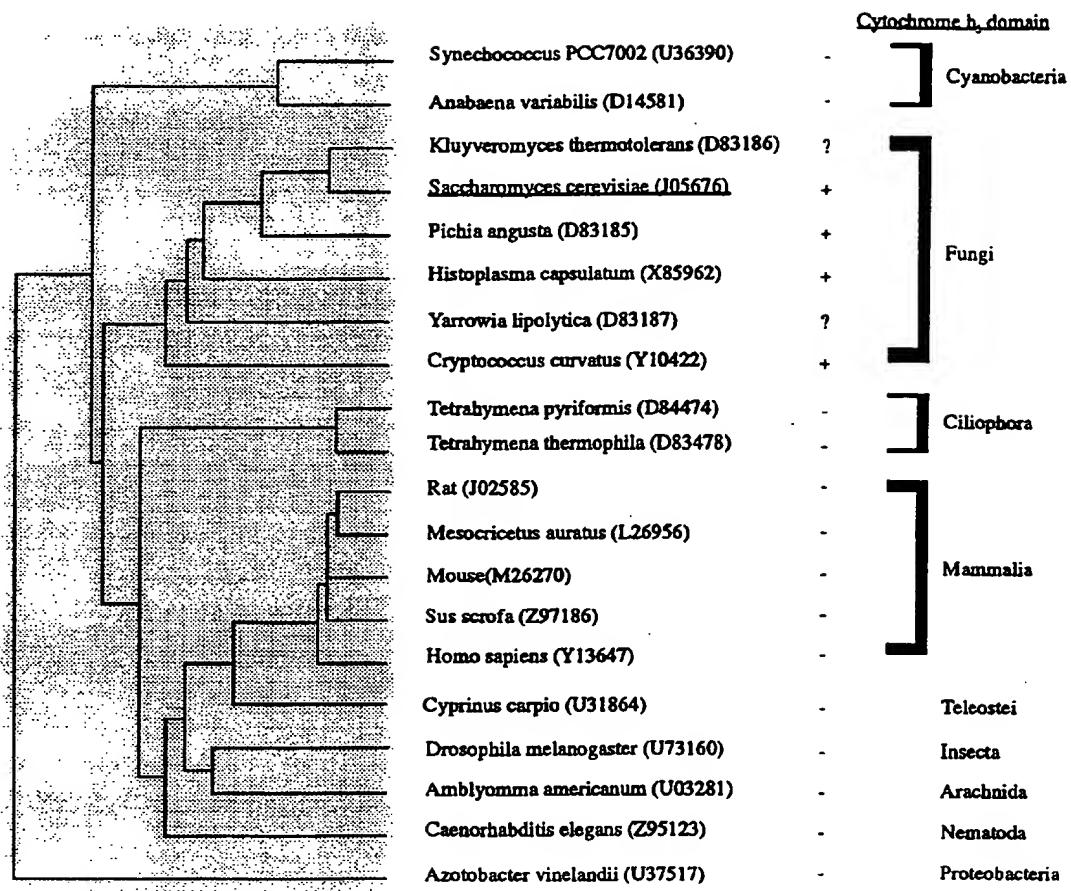


FIGURE 1

2/2

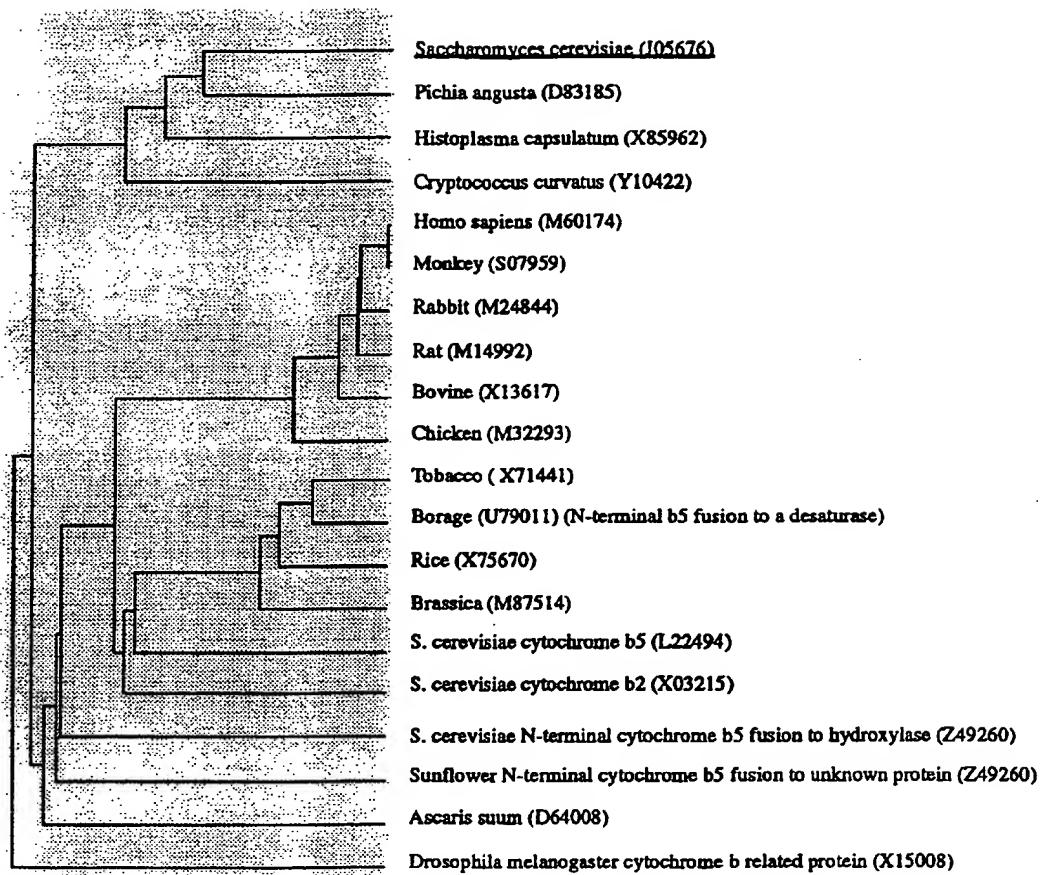


FIGURE 2

SEQUENCE LISTING

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 Mitchell, Andrew

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3

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/19443

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/02; C12N 15/82; A01H 5/00
US CL :536/23.1; 800/281, 298; 435/69.1, 419, 468

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 800/281, 298; 435/69.1, 419, 468

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST/DERWENT, GENBANK, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,777,201 A (POUTRE et al) 07 July 1998, especially col.14-18.	1-5,9-11,13-15
Y		6-8,12,16-21
Y	US 5,057,419 A (MARTIN et al) 15 October 1991, col. 7-11.	1-21
Y	STUKEY et al. The OLE1 Gene of <i>Saccharomyces cerevisiae</i> Encodes the delta 9 Fatty Acid Desaturase and Can Be Functionally Replaced by the Rat Stearoyl-CoA Desaturase. Gene. The Journal of Biological Chemistry. 25 November 1990, Vol. 265, No. 33, pages 20144-20149, especially page 20146.	1-21

Further documents are listed in the continuation of Box C.

See patent family annex.

•	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"g."	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
12 NOVEMBER 1999	06 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>D. Lawrence Fox</i> ELIZABETH F. MCELWAIN Telephone No. 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/19443

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	POLASHOCK et al. Expression of the Yeast delta-9 Fatty Acid Desaturase in <i>Nicotiana tabacum</i> . <i>Plant Physiology</i> . 1992, Vol. 100, pages 894-901, especially page 895.	1-5,9-11,13-15 ----- 6-8,12,16-21
Y		

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